Structural Biochemistry

DOI: 10.1002/anie.200703057

Structural "Snapshots" along Reaction Pathways of Non-Heme Iron Enzymes**

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bioinorganic chemistry · metalloenzymes · non-heme iron complexes · oxygen activation · superoxide reductase

Dioxygen and other reactive oxygen species react in numerous ways with biological non-heme iron centers to carry out a breadth of cellular processes. Although there have been many kinetic and spectroscopic studies of the interactions of iron with dioxygen and related reduced oxygen species,[1] there are only a few examples that provide detailed structural data for the coordination chemistry that may occur when dioxygen species interact with mononuclear non-heme iron centers. Recent reports in *Science* from Katona et al.[2] and Kovaleva and Lipscomb[3] added six unique species that derive from the interactions of O_2 or H_2O_2 with non-heme iron active sites, augmenting the growing list of structurally characterized biological dioxygen adducts. The addition of these complexes from *Brevibacterium fuscum* homoprotocatechuate 2,3-dioxygenase (HPCD) and *Desulfoarculus baarsii* superoxide reductase (SOR) variant E114A represents a near doubling of the number of structurally characterized dioxygen and reduced dioxygen adducts bound to mononuclear non-heme iron centers, and helps us to better visualize how dioxygen species interact with non-heme iron centers in biology. Through a number of efforts, a generalized mechanism for the reactions of dioxygen and superoxide with iron has been formulated (Figure 1).[1] The first stage of dioxygen activation occurs when O_2 coordinates to an iron(II) center to form an iron–O_2 adduct. This adduct is typically described as an iron(III)–superoxo species,[4] in which an electron from the iron center has been transferred to the dioxygen moiety. The next stage of dioxygen activation requires additional electrons to be acquired by the bound O_2 from either substrate or cofactor, such as α-ketoglutarate, tetrahydrobiopterin, or other redox centers, allowing the dioxygen species to be further reduced through the peroxo state, eventually leading to the cleavage of the O–O bond and the desired substrate oxidation. Alternatively, the bound superoxide may dissociate and introduce reactive oxygen species into the cell that can cause oxidative damage to various biological molecules.[5] Nature has developed several defense mechanisms to minimize the lifetime of superoxide within cells. One such strategy is to directly reduce $O_2^{-\bullet}$ to H_2O_2, a process catalyzed by superoxide reductase, a key player in the $O_2^{-\bullet}$ defense mechanism occurring in a variety of anaerobic bacteria and archaea.[1b,6] The mononuclear non-heme iron(II) center of SOR reacts with superoxide to form a putative iron(III)–peroxo species, whose protonation state has not been established.[1b]

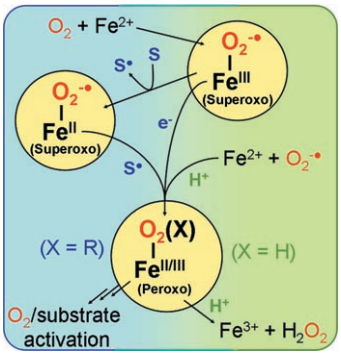

Figure 1. Intermediate species that have been observed at non-heme iron centers that are involved in O_2 activation and $O_2^{-\bullet}$ reduction. The conversion of S into S• reflects one-electron injection into the Fe(III)–$O_2^{-\bullet}$ moiety by the substrate in the HPCD reaction pathway.

In the *Science* article describing the O_2 activation mechanism used by aromatic ring cleaving dioxygenases, Kovaleva and Lipscomb reported a new X-ray crystal structure of HPCD with the substrate analogue 4-nitrocatechol (4-NC) bound to the iron center.[3] The crystal was grown in a low dioxygen atmosphere and solved to 1.95-Å resolution. Remarkably, three distinct species were observed in each homotetrameric packing arrangement, representing the 4-NC-bound iron site at different stages of its reaction with O_2. In subunit C, a side-on bound dioxygen species served to approximate the electron density about the 4-NC coordinated metal center, while in subunits B and D an alkylperoxo species was formulated to account for the electron density present around the respective iron centers. Finally, the 4-NC ring cleavage product modeled the electron density observed at the iron center of subunit A, implicating enzymatic turnover within this crystalline matrix.

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[**] Our work on non-heme iron enzymes is supported by the National Institutes of Health (GM-33126 for L.Q.).

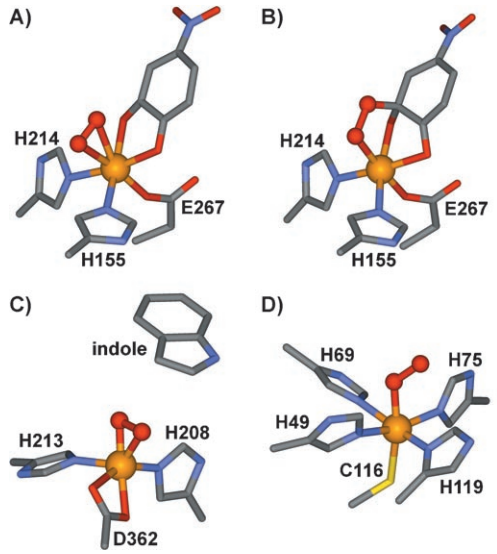

Figure 2. Active site structures of the O_2 adducts of several mononuclear non-heme iron enzymes structurally characterized by X-ray crystallography (orange Fe, red O, blue N, yellow S). A) The side-on O_2 adduct observed in subunit C of the *B. fuscum* HPCD–4-NC substrate complex crystal structure (2IGA.pdb). B) The end-on O_2 adduct observed in subunit B (an identical structure is present in subunit D) of the *B. fuscum* HPCD–4-NC substrate complex crystal structure (2IGA.pdb), illustrating attack of one atom of O_2 onto the 4-NC substrate. C) The side-on O_2 adduct observed in the crystal structure of *Pseudomonas* sp. NDO in the presence of an indole substrate (1O7N.pdb). D) The end-on H_2O_2 adduct observed in subunit B of the crystal structure of E114 A SOR from *D. baarsii* (2JI3.pdb). Similar structures are also observed in subunits C and D.

The species that Kovaleva and Lipscomb describe as an iron–superoxo species is shown in Figure 2 A. The side-on bound dioxygen species has an O–O bond length of approximately 1.3 Å and Fe–O bond lengths of 2.3–2.4 Å. It is positioned such that it appears to be poised to interact with the C2 carbon of the 4-NC moiety. This moiety found in HPCD can be directly compared to the Fe–O_2 adducts observed in four other crystallographically characterized non-heme iron enzymes listed in Table 1, including a related extradiol dioxygenase[7] and three members of the Rieske dioxygenase family.[8–11] Altogether these comprise seven unique iron–oxygen species with crystallographically established bond lengths listed in Table 1. Two structures show dioxygen bound in an end-on fashion, whereas three others have dioxygen bound in a side-on fashion. The structurally observed O–O bond lengths range from 1.2 to 1.4 Å, making the dioxygen oxidation state difficult to assign in these O_2 adducts because of the limited resolution of the protein crystal structures. In the case of the HPCD structure, however, it is clear that the bound 4-NC adopts a puckered, nonplanar conformation, with the C2 of 4-NC noticeably deviating from sp^2 hybridization, which is consistent with a one-electron oxidation of the catechol ring to its semiquinone form. The unequivocal loss of ring planarity for the bound 4-NC substrate moiety in the HPCD–4-NC–O_2 adduct of subunit C provides strong evidence for the initiation of electron transfer from 4-NC to O_2 in this complex, justifying the intermediacy of either an iron(II)–superoxo or iron(III)–peroxo moiety.

Kovaleva and Lipscomb propose this dioxygen adduct to be an iron(II)–superoxo species because of the observed Fe–O bond lengths of around 2.5 Å, which are notably longer than those found for other side-on bound O_2 adducts in Table 1. In particular, the η^2-O_2/indole complex of naphthalene dioxygenase (NDO), shown in Figure 2 C, has a much shorter average Fe–O bond length (1.9 Å), supporting its description as an iron(III)–peroxo species. The remaining Fe–η^2-O_2 adducts have average Fe–O bond lengths of 2.2–2.3 Å, intermediate between the NDO and HPCD extremes, suggesting an iron(III)-superoxo formulation for these adducts.

Subunits B and D of the HPCD-O_2/4-NC complex house an active site species further along the catalytic cycle, described as an iron–alkylperoxo species. This tridentate alkylperoxo species (Figure 2 B) is presumably formed from the combination of the reduced dioxygen moiety described above with the one-electron oxidized catechol ring. The O–O

Table 1: Crystallographic data for various iron–dioxygen and –reduced dioxygen species collected from their respective PDB files.[a]

Enzyme	Subunit	PDB file	Resolution [Å]	Description	*r*(Fe–O1)[b] [Å]	*r*(Fe–O2)[b] [Å]	*r*(O1–O2)[b] [Å]
HPCD[3]	C	2IGA	1.95	η^2-O_2/4-NC adduct 1	2.43	2.52	1.31
	B			O_2/4-NC adduct 2	2.22	2.81	1.53
	D			O_2/4-NC adduct 2	2.09	2.82	1.53
E114A SOR[2]	B	2JI3	1.95	η^1-H_2O_2 adduct	2.00	3.10	1.46
	C			η^1-H_2O_2 adduct	1.97	3.08	1.47
	D			η^1-H_2O_2 adduct	2.04	3.07	1.47
HADO[7]		1YFW	2.0	η^1-O_2/Cl-HAA adduct	2.05	3.22	1.20
BPDO[8]	A	2GBW	1.7	η^2-O_2 adduct	2.30	2.35	1.21
	C			η^2-O_2 adduct	2.20	2.36	1.20
	E			η^2-O_2 adduct	2.26	2.34	1.20
CumDO[9]		1WQL	2.2	η^1-O_2 adduct	2.07	2.70	1.23
NDO[10]		1O7M	1.95	η^2-O_2 adduct	2.15	2.34	1.45
		1O7N	1.4	η^2-O_2/indole adduct	1.73	1.98	1.46
SLO[12]		1IK3	2.0	product complex	2.01	2.79	1.22

[a] Abbreviations: 3-hydroxyanthranilate-3,4-dioxygenase (HADO), biphenyl 2,3-dioxygenase (BPDO), cumene dioxygenase (CumDO), naphthalene dioxygenase (NDO), 4-nitrocatechol (4-NC), 4-chloro-3-hydroxyanthranilate (Cl-HAA) [b] All distances measured from the coordinates in the protein data bank (PDB) files.

moiety is now bound in an end-on fashion, with an O–O bond length in these subunits of 1.5 Å, which led Kovaleva and Lipscomb to assign this moiety as an iron(II)–alkylperoxo species. The proximal oxygen (O1) atom remains within bonding distance of the iron center, whereas the distal oxygen (O2) atom is approximately 2.8 Å from the iron center and appears to be bound to the C2 carbon atom of what once was 4-NC. The only other end-on alkylperoxo species structurally observed in an enzyme is the enzyme–product complex of soybean lipoxygenase (SLO). The Fe–alkylperoxo species found in SLO has a comparable Fe–O1 bond length to that of the HPCD–O_2/4-NC putative alkylperoxo species. However the O–O bond length of 1.2 Å found in the SLO structure is curiously short.[12]

The work of Kovaleva and Lipscomb thus provides us with unique three-dimensional "snapshots" along the course of dioxygen activation occurring in the active site of HPCD. These structures substantiate mechanistic ideas associated with substrate-initiated dioxygen activation in non-heme iron enzymes first proposed over 30 years ago.[13] Additional spectroscopic investigations into the electronic nature of these crystallographically observed intermediate species should prove insightful in formalizing the mechanism for the aromatic ring-cleaving dioxygenases, such as HPCD.

The second *Science* article describes the structure of an H_2O_2 adduct of a variant superoxide reductase that is presumably related to the corresponding species formed upon superoxide binding.[2] In this case, however, the authors also provide relevant spectroscopic evidence collected in the crystal, strengthening their position on the mechanism of superoxide reduction.

The details of the SOR reaction mechanism have been debated on the basis of somewhat differing results.[1b] Kurtz and co-workers initially proposed an iron(III)–(hydro)peroxo intermediate species after observing an intense visible chromophore ($\lambda_{max} = 650$ nm, $\varepsilon_{650} \approx 1500\,M^{-1}cm^{-1}$) produced stoichiometrically from the reaction of superoxide with iron(II)–SOR.[14] In 2002 an iron(III)–peroxo species formed from a mutant SOR by a H_2O_2 shunt pathway was observed by electronic absorption spectroscopy, and characterized by EPR and resonance Raman spectroscopy.[15] Oxygen-isotope-sensitive resonance Raman vibrations at 850 and 438 cm^{-1} were described and led the authors to propose a side-on peroxo unit, although the key mixed-isotope experiment necessary to establish this binding mode definitively was not described.

Presently, the crystallographic evidence reported by Katona et al. on a proposed iron(III)–(hydro)peroxo species formed at the active center of SOR provides us with an unprecedented opportunity to see the possible orientations of an end-on iron–peroxo species in this enzyme.[2] In this report, the authors describe three structurally distinct iron–peroxo complexes formed in three of the four subunits of the crystallographic homotetramer. Notably, these peroxo complexes were formed by a hydrogen peroxide shunt-type mechanism, whereby reduced SOR crystals were anaerobically incubated in a solution of hydrogen peroxide. Although this may not be the same intermediate species as that formed under oxidative stress in the presence of superoxide, these structures do provide insight into how peroxo intermediates formed from $O_2^{-\bullet}$ may look and act. The three iron–peroxo species observed in the homotetrameric structure are all bound in an end-on fashion with Fe–O1 and Fe–O2 bond lengths ranging from 1.9 to 2.0 Å and 3.0 to 3.1 Å, respectively. The O–O bond length in all of these transient species is around 1.4–1.5 Å. Several residues were also identified that may be involved in hydrogen bonding with the iron–peroxo unit.

To augment the crystallographic results in this study, the authors developed a Raman spectrometer capable of collecting data in the crystal. They describe oxygen-isotope-sensitive Raman bands at 838 and 567 cm^{-1}, which support the assignment of this feature as an end-on iron(III)–peroxo species. The latter vibration associated with the $\nu(Fe^{III}–OOH)$ mode is quite different from that observed at 438 cm^{-1} in the earlier study,[15] consistent with the proposed differences in the binding mode of the peroxo ligand, which are precedented in the spectra of synthetic iron–peroxo complexes.[1a,16] Clearly, different peroxo intermediates are formed in solution and in the crystal. The combination of protein X-ray crystallography with vibrational spectroscopic techniques has thus afforded both structural geometric parameters and insights into oxidation states, clarifying how a peroxo species may be bound to the non-heme iron center of SOR.

In closing, these elegant crystallographic studies of HPCD and SOR provide unique structural data for the interaction of dioxygen and its reduced congeners with iron centers within a protein milieu. Moreover, they are a beautiful illustration of how recent advances in protein crystallographic techniques have provided new information concerning the interactions of dioxygen with non-heme iron centers.[17] These results will undoubtedly invigorate the study of dioxygen moieties and their iron coordination chemistry.

Published online: October 8, 2007

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