



Formation of High-Valent Iron–Oxo Species in Superoxide Reductase: Characterization by Resonance Raman Spectroscopy**

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Abstract: Superoxide reductase (SOR), a non-heme mononuclear iron protein that is involved in superoxide detoxification in microorganisms, can be used as an unprecedented model to study the mechanisms of O_2 activation and of the formation of high-valent iron–oxo species in metalloenzymes. By using resonance Raman spectroscopy, it was shown that the mutation of two residues in the second coordination sphere of the SOR iron active site, K_{48} and I_{118} , led to the formation of a high-valent iron–oxo species when the mutant proteins were reacted with H_2O_2 . These data demonstrate that these residues in the second coordination sphere tightly control the evolution and the cleavage of the O–O bond of the ferric iron hydroperoxide intermediate that is formed in the SOR active site.

The activation of molecular oxygen by metalloenzymes is an essential process in biological systems that enables the catalysis of a wide variety of vital oxidation reactions.^[1] The formation of a high-valent iron–oxo unit through the cleavage of the O–O bond of an Fe^{III} –OOH species is a key step in the catalytic cycle of several dioxygen-activating enzymes, and the mechanisms through which the protein environment tightly controls these processes have a central importance for these systems.^[1] Whereas studies on synthetic iron complexes have provided detailed information on the chemistry of these metal–oxide species, direct experimental data on metalloenzymes are more scarce, as trapping and identification of reaction intermediates have remained difficult.^[1]

Interestingly, superoxide reductase (SOR), which is a small non-heme iron protein that is not involved in oxidation reactions, but in superoxide radical ($O_2^{\cdot-}$) detoxification in microorganisms,^[2] presents at least two striking similarities to cytochrome P450 oxygenase (Figure 1).^[1b,c]

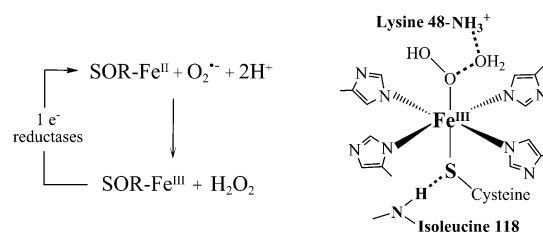


Figure 1. SOR detoxification activity and schematic representation of its ferric iron active site bound to a peroxide, as determined from its crystal structure (PDB ID: 2ji3).^[3a] Hydrogen bonds are shown as dotted lines.

First, both enzymes contain a mononuclear iron site with an $\{FeN_4S\}$ square-pyramidal pentacoordinate iron center and a cysteine ligand in the axial position. Second, the catalytic cycles of both SOR^[2c,e,f,3] and cytochrome P450^[1c] involve an Fe^{III} –OOH intermediate (compound 0 in P450). However, unlike cytochrome P450, SOR does not cleave the O–O bond of the Fe^{III} –OOH unit to generate a high-valent iron–oxo species, which is responsible for the oxidation reactions,^[1b–d] but rather cleaves the Fe–O bond to form its reaction product H_2O_2 .^[2c,e,f,3]

Hence, SOR could be an attractive model to depict the mechanisms by which a polypeptide chain controls the evolution of an Fe^{III} –OOH intermediate. Although it was hypothesized that the differences in the reactivity between SOR and cytochrome P450 could be ascribed to the nature of the equatorial nitrogen ligands (four histidine residues in SOR versus a porphyrin ring in cytochrome P450) and to the spin state of the Fe^{III} –OOH species (high spin for SOR vs. low spin for cytochrome P450),^[4] recent studies have suggested that the residues that are present in the second coordination sphere might play more decisive roles.^[5] In fact, a mutation of the second-coordination-sphere residue K_{48} into isoleucine was proposed to cancel the specific protonation of the proximal oxygen atom of the Fe^{III} –OOH intermediate (Figure 1),^[3a] which might lead to the formation of an iron–oxo species.^[5a] Another recently characterized SOR mutant, $I_{118}S$, which entailed a weakened hydrogen bond between the I_{118} N–H moiety of the main chain of the peptide and the sulfur atom of the cysteine ligand (Figure 1), might also modify the

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evolution of the $\text{Fe}^{\text{III}}\text{-OOH}$ intermediate and favor the formation of iron-oxo species.^[5b]

Herein, we show by using resonance Raman (RR) spectroscopy that the reaction of both the I_{118}S and K_{48}I SOR mutants with H_2O_2 leads to the formation of a high-valent iron-oxo species in their active sites. These data demonstrate that in SOR, the evolution of the $\text{Fe}^{\text{III}}\text{-OOH}$ intermediate and the cleavage of the Fe-O bond instead of the O-O bond are tightly controlled by at least two different second-coordination-sphere residues, that is, K_{48} and I_{118} .

RR spectroscopy was carried out on the I_{118}S SOR mutant that had been reacted with H_2O_2 at an excitation wavelength of 647.1 nm (for experimental details, see the Supporting Information). These conditions are similar to those of previous RR investigations that showed the formation of $\text{Fe}^{\text{III}}\text{-OOH}$ species in the E_{47}A and E_{114}A SOR mutants.^[3b,c] When the I_{118}S mutant (with a ferrous iron active site) was rapidly mixed with 2–4 equivalents of H_2O_2 and frozen in liquid N_2 within five seconds of incubation time, RR spectroscopy at 15 K indicated the presence of bands in the 300 cm^{-1} region and at 742 cm^{-1} ; these bands were previously associated to modes of the $\text{Fe}^{\text{III}}\text{-S}(\text{Cys})$ moiety and of the internal cysteine ligand, respectively (Figure 2A).^[6] Accordingly, these RR bands were not sensitive to isotopic labeling after reaction with $\text{H}_2^{18}\text{O}_2$ (Figure 2A).

Two other bands were observed at 826 and 845 cm^{-1} (Figure 2A and B). These two bands could not be attributed to a $\nu(\text{O-O})$ stretching mode of an iron(III)–(hydro)peroxo species, as previously observed for the E_{47}A ^[3b] and E_{114}A ^[3c] SOR mutants after the reaction with H_2O_2 , as no ^{18}O -sensitive band that may be assigned to a $\nu(\text{Fe-O})$ stretching mode was detected in the $440\text{--}450\text{ cm}^{-1}$ region (Figure 2A). The origin of the minor band at 845 cm^{-1} is difficult to assign as its isotopic sensitivity was not clearly seen (Figure 2B). On the contrary, the dominant band at 826 cm^{-1} exhibited a shift by -33 cm^{-1} upon $^{16}\text{O}/^{18}\text{O}$ substitution (793 cm^{-1} ; Figure 2A and B). This value is much lower than that expected for a pure $\nu(\text{O-O})$ stretching mode of an iron(III)–(hydro)peroxo species (-48 cm^{-1}).^[3b,c] Instead, the line at 826 cm^{-1} is consistent with a $\nu(\text{Fe=O})$ stretching mode of a high-valent iron-oxo species that is formed in the active site of SOR. Such species have been well described for several non-heme iron complexes of tetramethylcyclam (TMC) and related macrocyclic ligands,^[1e,f] as well as for the TauD oxygenase,^[7] with $\nu(\text{Fe=O})$ modes in the $810\text{--}860\text{ cm}^{-1}$ range. Accordingly, the experimentally determined shift of the 826 cm^{-1} band by -33 cm^{-1} that we observed (Figure 2) is very close to the theoretical values assuming a simple linear three-body S-Fe=O oscillator model for the axial ligation (-34.6 to -35.1 cm^{-1}).^[8,9] Furthermore, the $[\text{Fe}^{\text{II}}(\text{TMC})(\text{NCS})]$ complex was shown to form an $\text{Fe}^{\text{IV}}\text{=O}$ species with a $\nu(\text{Fe-O})$ vibration at 820 cm^{-1} and an ^{18}O isotopic shift value of -34 cm^{-1} .^[10] These values are very close to those described here for the I_{118}S SOR mutant.

Electronic spectra of various Fe=O complexes have been reported to exhibit low-intensity near-IR features, with absorption maximum wavelengths ranging from 750 to 1000 nm ($\epsilon = 100\text{--}400\text{ M}^{-1}\text{ cm}^{-1}$).^[1e,f] As shown in Figure S1, the absorbance spectrum that results from the reaction of the

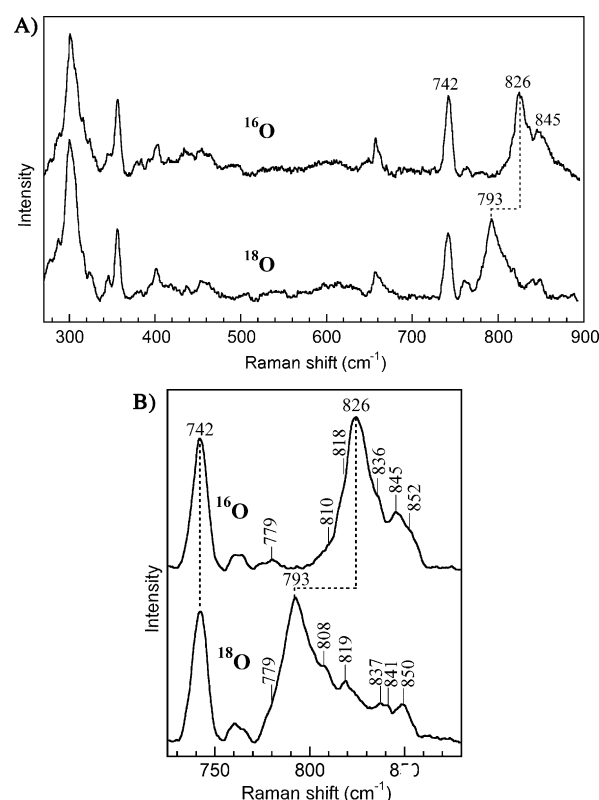


Figure 2. Resonance Raman spectra of the I_{118}S SOR mutant from *D. baarsii* (4 mM in 50 mM Tris/HCl, pH 8.5) excited at 647.1 nm (30 mW) at 15 K. The SOR solution (ferrous form) was treated with four equivalents of H_2O_2 , rapidly mixed, and frozen in liquid N_2 after an incubation time of five seconds at 5°C . Longer incubation times for the I_{118}S SOR mutant with H_2O_2 (30 s) did not induce noticeable modifications of the RR spectra. A: summation of 8 scans; B: summation of 30 scans. Upper spectra: reaction with $\text{H}_2^{16}\text{O}_2$; lower spectra: reaction with $\text{H}_2^{18}\text{O}_2$.

I_{118}S SOR mutant with H_2O_2 exhibited a broad band centered at 650 nm , indicating the presence of ferric iron active sites in solution.^[5b] This is in agreement with the observation of modes that are due to the $\text{Fe}^{\text{III}}\text{-S}(\text{Cys})$ moiety in the 300 cm^{-1} region of the RR spectra (Figure 2A). However, a comparison of this absorbance spectrum with that of an I_{118}S SOR mutant that was oxidized with $\text{Ir}^{\text{IV}}\text{Cl}_6$ (which only generates the ferric iron form) revealed the formation of additional bands in the $750\text{--}950\text{ nm}$ region when the protein was oxidized with H_2O_2 (Supporting Information, Figure S1). These weak near-infrared bands could originate from the Fe=O species formed in SOR. The enhancement of the RR signal with an excitation wavelength of 647.1 nm suggests that absorbance bands that are associated with the Fe=O species in SOR are also present in the 650 nm region. These bands and those of the ferric iron species should be superimposable (Figure S1). Nevertheless, as the relative amounts of the ferric iron and Fe=O species formed under these conditions could not be precisely specified, the overall absorbance spectrum of the Fe=O species formed in SOR could not be determined.

When the K_{48}I SOR mutant (with its iron active site in a ferrous form) was rapidly mixed with an excess of H_2O_2 and frozen in liquid N_2 after an incubation time of five seconds,

the RR spectra recorded at 15 K exhibited two major bands at 444 and 850 cm^{-1} , which were downshifted to 424 (-20) and 802 (-48) cm^{-1} , respectively, after mixing with $\text{H}_2^{18}\text{O}_2$ (Figure 3 A and B). The frequencies of these RR bands as well as their sensitivities towards ^{18}O isotopic labeling are consistent with the $\nu(\text{Fe}-\text{O})$ and $\nu(\text{O}-\text{O})$ assignments for an iron(III)–(hydro)peroxo species, as described previously for the $\text{E}_{47}\text{A}^{[3b]}$ and $\text{E}_{114}\text{A}^{[3c]}$ mutants. Similarly to the I_{118}S mutant (Figure 2 A), the bands in the 300 cm^{-1} region and at 742 cm^{-1} can be assigned to vibrational modes of the $\text{Fe}^{\text{III}}-(\text{S}-\text{C}(\text{Cys}))$ group. The band at 867 cm^{-1} , which was downshifted to 821 cm^{-1} (-46 cm^{-1}) after mixing with $\text{H}_2^{18}\text{O}_2$ (Figure 3 B), could be attributed to H_2O_2 that is weakly bound to the ferric iron center,^[11] taking into account that the formation of this complex could be favored by the large excess of hydrogen peroxide used with this mutant. Interestingly, an additional band at 826–828 cm^{-1} was detected (Figure 3 A and B), which was not observed in the RR spectra that were recorded after the reaction of the $\text{E}_{47}\text{A}^{[3b]}$ and $\text{E}_{114}\text{A}^{[3c]}$ mutants with H_2O_2 . This band at 826–828 cm^{-1} appeared to be ^{18}O -sensitive and possibly downshifted to 794 cm^{-1} to appear as a noticeable shoulder of the dominant 802 cm^{-1} band (Figure 3 B).

Longer incubation times for the K_{48}I mutant with $\text{H}_2^{16}\text{O}_2$ (30 s) before freezing led to an increase in intensity of the feature at 826 cm^{-1} , compared to the bands at 850, 742, and 444 cm^{-1} (Figure 3 C and D, upper spectra). After mixing with $\text{H}_2^{18}\text{O}_2$, the band at 826 cm^{-1} shifted to 795 cm^{-1} (by -31 cm^{-1} ; Figure 3 D, lower spectra). Compared to the spectra that were obtained after an incubation time of five seconds, no modification of the other RR bands was observed (Figure 3 A and C). Similarly to the 826 cm^{-1} line of the I_{118}S mutant (Figure 2), the 826 cm^{-1} band of the K_{48}I mutant can be attributed to a $\nu(\text{Fe}=\text{O})$ stretching mode of a high-valent iron–oxo species. However, formation of the $\text{Fe}=\text{O}$ species in the K_{48}I mutant was slower, which is in agreement with the increase in intensity of the 826 cm^{-1} band when the incubation times was extended from 5 to 30 seconds (Figure 3 B and D). Interestingly, formation of the $\text{Fe}^{\text{III}}-\text{OOH}$ species in the K_{48}I mutant mainly occurred before formation of the iron–oxo species. These data suggest that for the K_{48}I mutant, the $\text{Fe}=\text{O}$ species was formed from the $\text{Fe}^{\text{III}}-\text{OOH}$ species. The fact that the intensities of the 444 and 850 cm^{-1} RR bands remain constant when the incubation time was changed from 5 to 30 seconds (Figure 3 A and C) could reflect a steady-state concentration of the $\text{Fe}^{\text{III}}-\text{OOH}$ species, resulting from its formation and subsequent conversion into the $\text{Fe}=\text{O}$ species. These results are consistent with a mechanism that involves cleavage of the O–O bond of the $\text{Fe}^{\text{III}}-\text{OOH}$ intermediate to form the iron–oxo species, instead of cleavage of the Fe–O bond in wild-type SOR to form H_2O_2 .^[2,3] These data are in line with the X-ray structure of the SOR $\text{Fe}^{\text{III}}-\text{OOH}$ intermediates,^[3a] which suggested that the K_{48} residue could be involved in the specific protonation of the proximal oxygen atom of the intermediate, leading to Fe–O bond cleavage. In the K_{48}I mutant, such a specific protonation process cannot occur, and therefore, O–O bond cleavage of the $\text{Fe}^{\text{III}}-\text{OOH}$ intermediate should be favored.^[5a]

For the I_{118}S mutant, formation of the iron–oxo species could also result from the cleavage of the O–O bond of an

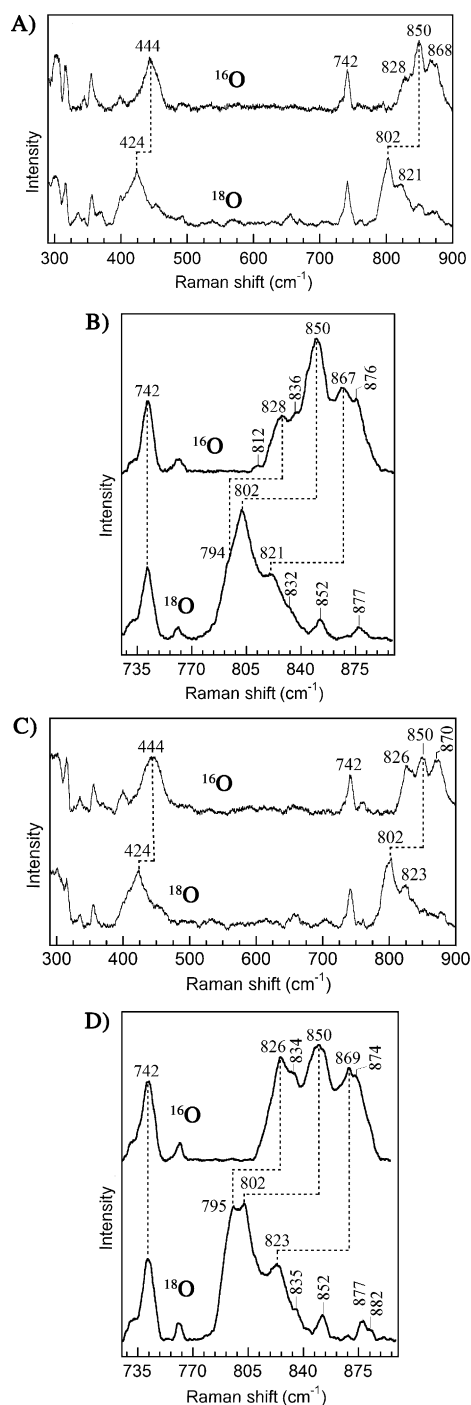


Figure 3. Resonance Raman spectra of the K_{48}I SOR mutant from *D. baarsii* (4 mM in 50 mM Tris/HCl, pH 8.5) excited at 647.1 nm (30 mW) at 15 K. The SOR solution (ferrous form) was treated with 30 equivalents of H_2O_2 , rapidly mixed, and frozen in liquid N_2 after incubation times of either 5 s (A and B) or 30 s (C and D) at 5 °C. A and C: summation of 8–9 scans; B and D: summation of 30 scans; the contribution of free H_2O_2 was subtracted. Upper spectra: reaction with $\text{H}_2^{16}\text{O}_2$; lower spectra: reaction with $\text{H}_2^{18}\text{O}_2$.

$\text{Fe}^{\text{III}}-\text{OOH}$ intermediate. Nevertheless, the faster rate of formation of the iron–oxo species that was observed for this mutant could explain the absence of an RR signal of the $\text{Fe}^{\text{III}}-\text{OOH}$ species (Figure 2). The I_{118}S mutation was

recently shown to impair a hydrogen bond between the I₁₁₈ N–H moiety of the main chain of the peptide and the sulfur atom of the cysteine ligand (Figure 1), which leads to a strengthening of the S–Fe bond.^[5b] When the I₁₁₈S mutant reacted with O₂^{•−}, the strengthening of the S–Fe bond induced an increase in the pK_a value of the first reaction intermediate, which was proposed to be a ferrous iron superoxo species.^[5a,b] In line with the observation that was made for the ferrous iron superoxo intermediate, it is conceivable that the I₁₁₈S mutation could also modify the pK_a value of the Fe^{III}–OOH species that is formed by the reaction with H₂O₂. Such a modification of the pK_a value might favor protonation of the distal oxygen atom of the Fe^{III}–OOH species, which leads to cleavage of the O–O bond and formation of an Fe=O species in the I₁₁₈S mutant. Indeed, the conversion of a high-spin TMC Fe^{III}–OOH complex into an iron–oxo species through O–O bond cleavage was recently shown to be facilitated by protonation of the distal oxygen atom.^[12,13]

Alternatively, in the I₁₁₈S mutant, the absence of a hydrogen bond on the thiolate ligand and the consequent increase in electron density on the iron atom^[5b] could lead to the stabilization of a high-valent iron–oxo species in the active site. Therefore, in this mutant, the formation of an Fe=O species may be favored compared to the wild-type protein. Further studies, in particular DFT QM/MM calculations, will be required to elucidate the mechanism through which the I₁₁₈S mutation leads to the formation of an Fe=O species.

Altogether, these data underline that in SOR, the fate of the Fe^{III}–OOH intermediate is not decisively controlled by its high-spin state and its equatorial histidyl ligands, but by the presence of specific residues in the second coordination sphere, namely K₄₈ and I₁₁₈ (Figure 4). We showed that these two residues, possibly by acting either through specific protonation of the proximal oxygen atom of the Fe^{III}–OOH species or by controlling the electron density on the sulfur ligand, prevent the formation of the unwanted iron–oxo species in the wild-type SOR.

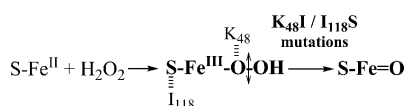


Figure 4. The formation of Fe=O species in the I₁₁₈S and K₄₈I SOR mutants.

In summary, we have reported the first direct evidence that the non-heme mononuclear {FeN₄S₁} site of superoxide reductase can accommodate a high-valent iron–oxo species (Figure 4). Up to now, the roles of second-coordination-sphere ligands in oxygen activation processes have been hardly documented for synthetic iron complexes and cytochrome P450; for the latter, one of its reaction intermediates, compound I, was directly characterized only very recently.^[1d] This work illustrates the fact that SOR may be a valuable model system to study the mechanisms of oxygen activation and formation of iron–oxo species in metalloenzymes

(Figure 4). Moreover, the fact that different SOR mutants can specifically generate either Fe^{III}–OOH or Fe=O species upon the reaction with H₂O₂ provides an unprecedented tool to investigate the reactivity of each of these species in non-heme metalloenzymes. The oxidation properties of the different SOR mutants are currently under investigation.

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