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# Formation of High-Valent Iron-Oxo Species in Superoxide Reductase. Characterization by Resonance Raman Spectroscopy\*\*

Florence Bonnot, Emilie Tremey, David von Stetten, Stéphanie Rat, Simon Duval, Philippe Carpentier, Martin Clemancey, Alain Desbois, and Vincent Nivière\*

**Abstract:** In this work, we report that Superoxide Reductase (SOR), a non-heme mononuclear iron protein involved in superoxide detoxification in microorganisms, can be used as an unprecedented model to study the mechanisms of  $O_2$  activation and formation of high-valent iron-oxo species in metalloenzymes. Using resonance Raman spectroscopy, we show that the mutation of two second coordination sphere residues of the SOR iron active site, K48 and I118, leads 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 second coordination sphere residues tightly control the evolution and the cleavage of the O-O bond of the ferric iron hydroperoxide intermediate formed in the SOR active site.

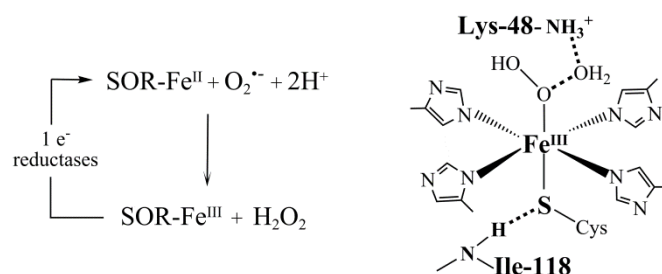
Activation of molecular oxygen by metalloenzymes is an essential process in biological systems, allowing the catalysis of a wide variety of vital oxidation reactions.<sup>[1]</sup> Formation of a high-valent iron-oxo unit from the cleavage of the O-O bond of  $Fe^{III}$ -OOH species is a key step in the catalytic cycle of several  $O_2$ -activating enzymes and the mechanisms by which the protein environment tightly controls these processes represent a central question for these systems.<sup>[1]</sup> 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, since trapping and identification of reaction intermediates remain difficult.<sup>[1]</sup>

Interestingly, superoxide reductase (SOR), which is a small non-heme iron protein not involved in oxidation reactions but in superoxide radical ( $O_2^{\bullet-}$ ) detoxification in microorganisms,<sup>[2]</sup> presents at least two striking similarities with cytochrome P450 oxygenase (Figure 1).<sup>[1b,c]</sup> First, both enzymes contain a mononuclear iron site with a  $[FeN_4S_1]$  square pyramidal pentacoordination and a cysteine ligand in the axial position. Second, SOR<sup>[2c,e,f,3]</sup> and cytochrome P450<sup>[1c]</sup> involve an  $Fe^{III}$ -OOH intermediate (compound 0 in P450) in their catalytic cycle. 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, responsible for the oxidation reactions,<sup>[1b-d]</sup> but rather cleaves the Fe-O bond to form its reaction product  $H_2O_2$ .<sup>[2c,e,f,3]</sup>

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 versus low-spin for cytochrome P450),<sup>[4]</sup> recent studies suggested that the presence of second coordination sphere residues might play more determining roles.<sup>[5]</sup> In fact, mutation of the second coordination sphere residue K48 into Ile was proposed to cancel the specific protonation of the proximal oxygen of the  $Fe^{III}$ -OOH intermediate (Figure 1),<sup>[3a]</sup> which might lead to the formation of an iron-oxo species.<sup>[5a]</sup> Another recently characterized SOR mutant, I118S, which weakened a H-bond between the I118 NH

peptide main chain and the sulfur atom of the cysteine ligand (Figure 1), might also affect the evolution the  $Fe^{III}$ -OOH intermediate and favour formation of iron-oxo species.<sup>[5b]</sup>

In this work, using resonance Raman (RR) spectroscopy, we show that the reaction of both the I118S and K48I 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  $Fe^{III}$ -OOH intermediate and the cleavage of the Fe-O bond instead the O-O one is tightly controlled by, at least, two different second coordination sphere residues, K48 and I118.

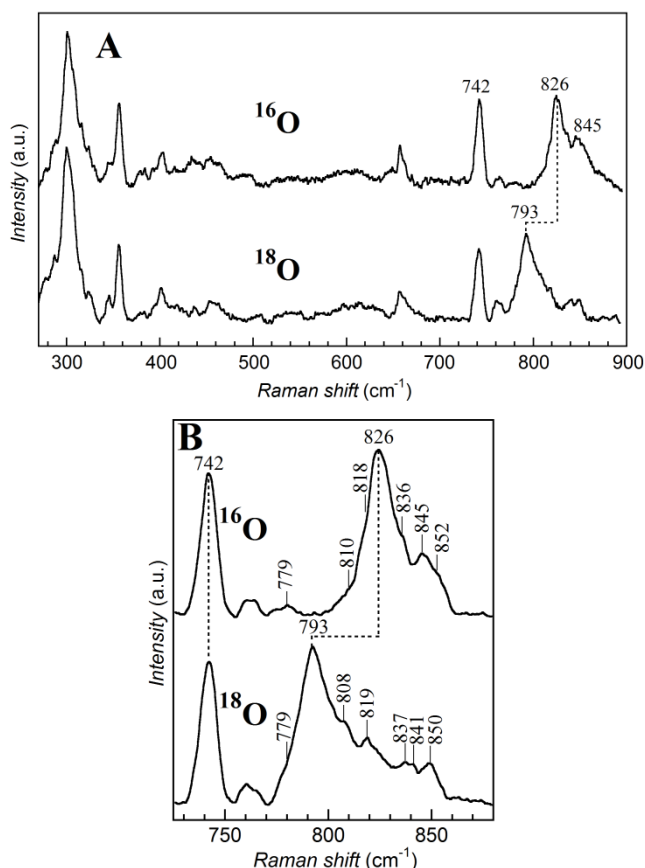


**Figure 1.** SOR detoxification activity and schematic representation of its ferric iron active site bound to a peroxide, from its crystal structure (PDB ID: 2ji3<sup>[3a]</sup>). Hydrogen bonds are shown as dotted lines.

Using a 647.1 nm-excitation, RR spectroscopy was carried out on the I118S SOR mutant reacted with  $H_2O_2$  (for experimental details, see Supporting Information). These conditions are similar to previous RR investigations that identified the formation of  $Fe^{III}$ -OOH species in the E47A and E114A SOR mutants.<sup>[3b,c]</sup> As shown in Figure 2A, when the I118S mutant (with its iron active site in a ferrous form) was rapidly mixed with 2-4 equivalents  $H_2O_2$  and frozen in liquid  $N_2$  within 5 sec of incubation time, RR spectra recorded at 15 K indicated the presence of bands in the  $300\text{ cm}^{-1}$  region and at  $742\text{ cm}^{-1}$ , previously associated to  $Fe^{III}$ -S(Cys) involving-modes and to an internal cysteine ligand mode, respectively.<sup>[6]</sup> Accordingly, no isotopic sensitivity was observed for these RR bands after reaction with  $H_2^{18}O_2$  (Figure 2A).

Two other bands were observed at  $826$  and  $845\text{ cm}^{-1}$  (Figures 2A and 2B). These two bands could not be attributed to a  $\nu(O-O)$  stretching mode of an  $Fe^{III}$ -(hydro)peroxo species, as observed previously for the E47A<sup>[3b]</sup> and E114A<sup>[3c]</sup> SOR mutants reacted with  $H_2O_2$ , since no  $^{18}O$ -sensitive band assignable to a  $\nu(Fe-O)$  stretching mode was detected in the  $440\text{--}450\text{ cm}^{-1}$  region (Figure 2A). The origin of the minor band at  $845\text{ cm}^{-1}$  is difficult to assign since its isotopic sensitivity is not clearly seen (Figure 2B). On the contrary, the dominant band at  $826\text{ cm}^{-1}$  exhibited a  $-33\text{ cm}^{-1}$  shift upon  $^{16}O/^{18}O$  substitution ( $793\text{ cm}^{-1}$ ) (Figures 2A and 2B). This value is much lower than that expected for a pure  $\nu(O-O)$  stretching mode of an  $Fe^{III}$ -(hydro)peroxo species ( $-48\text{ cm}^{-1}$ ).<sup>[3b,c]</sup> Rather, the  $826\text{ cm}^{-1}$  line is consistent with a  $\nu(Fe=O)$  stretching mode of a high-

valentiron-oxo species formed in the active site of SOR. Such a species has been well described for several non-heme iron complexes of tetramethylcyclam (TMC) and related macrocyclic ligands,<sup>[1e,f]</sup> as well as for the TauDxygenase,<sup>[7]</sup> with  $\nu(\text{Fe=O})$  modes in the 810-860  $\text{cm}^{-1}$  range. Accordingly, the  $-33 \text{ cm}^{-1}$  experimental shift of the 826  $\text{cm}^{-1}$  band observed here (Figure 2) is very close to the theoretical values assuming a simple linear three-body S-Fe=O oscillator for the axial ligation ( $-34.6$  -  $-35.1 \text{ cm}^{-1}$ ).<sup>[8,9]</sup> 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  $\text{cm}^{-1}$  and an  $^{18}\text{O}$  isotopic shift value of  $-34 \text{ cm}^{-1}$ .<sup>[10]</sup> These values are very close to those reported here for the I118S SOR mutant.



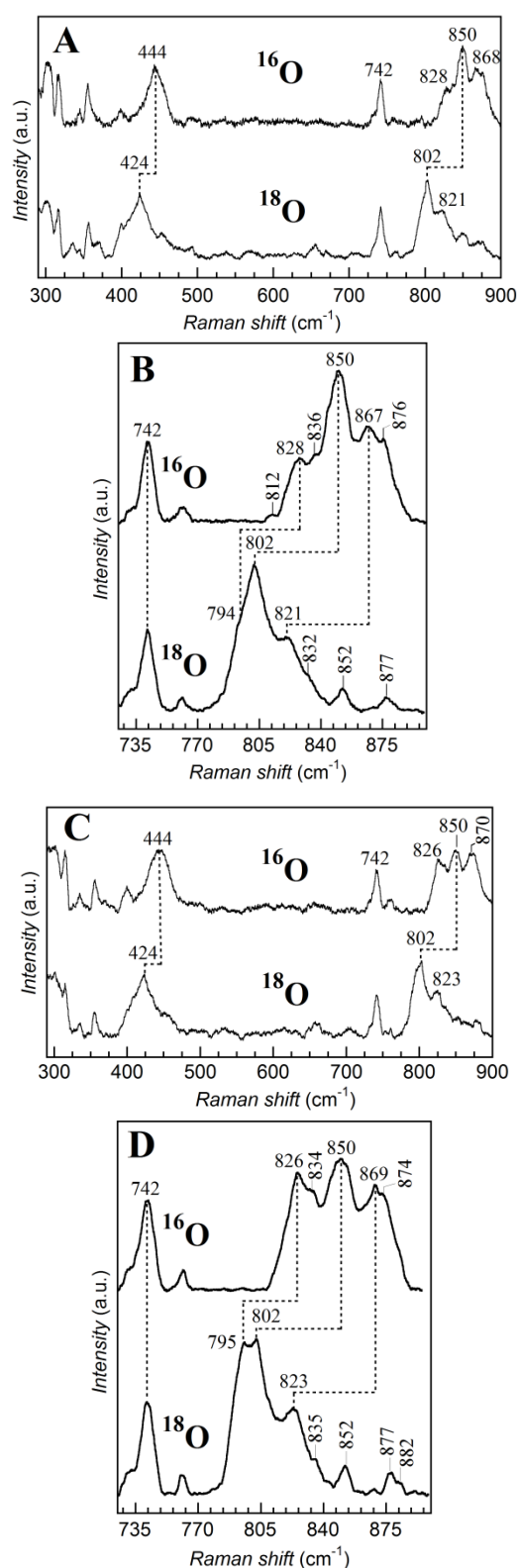
**Figure 2.** Resonance Raman spectra of the I118S SOR mutant from *D. baarsii* (4 mM in 50 mM Tris/HCl, pH 8.5) excited at 647.1 nm (30 mW) at 15 K. The SOR solution (ferrous form) was treated with 4 equivalents of  $\text{H}_2\text{O}_2$ , rapidly mixed and frozen in liquid  $\text{N}_2$  after 5 sec incubation time, at 5 °C. Longer incubation time of the I118S SOR mutant with  $\text{H}_2\text{O}_2$  (30 sec) 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  $\text{H}_2^{18}\text{O}_2$ .

Electronic spectra of various Fe=O complexes were reported to exhibit low intensity near-IR features, with absorption maximum wavelengths ranging from 750 to 1000 nm ( $\epsilon = 100\text{-}400 \text{ M}^{-1} \text{ cm}^{-1}$ ).<sup>[1e,f]</sup> As shown in Figure S1, the absorbance spectrum resulting from the reaction of the I118S SOR mutant with  $\text{H}_2\text{O}_2$  exhibited a broad band centered at 650 nm, indicating the presence of ferric iron active sites in the solution.<sup>[5b]</sup> This is in agreement with the observation of the  $\text{Fe}^{\text{III}}\text{-S}(\text{Cys})$  involving modes in the 300  $\text{cm}^{-1}$  region of the RR spectra (Figure 2A). However, comparison of this absorbance spectrum with that of a I118S SOR mutant oxidized with  $\text{Ir}^{\text{IV}}\text{Cl}_6$  (which only generates the ferric iron form) revealed formation of additional bands in the 750-950 nm region when the

protein was oxidized with  $\text{H}_2\text{O}_2$  (Figure S1). These weak near infrared bands could originate from the Fe=O species formed in SOR. Observation of RR enhancement with the 647.1 nm-excitation suggests that absorbance bands associated with the Fe=O species in SOR are also present in the 650 nm region. These bands should superimpose to those of the ferric iron (Figure S1). Nevertheless, since the relative amounts of the ferric iron and Fe=O species formed in these conditions could not be precisely specified, the whole absorbance spectrum of the Fe=O species formed in SOR could not be determined.

When the K48I SOR mutant (with its iron active site in a ferrous form) was rapidly mixed with an excess of  $\text{H}_2\text{O}_2$  and frozen in liquid  $\text{N}_2$  within 5 sec incubation time, the RR spectra recorded at 15 K exhibited two major bands at 444 and 850  $\text{cm}^{-1}$ , downshifting to 424 (-20) and 802 (-48)  $\text{cm}^{-1}$ , respectively, after mixing with  $\text{H}_2^{18}\text{O}_2$  (Figures 3A and 3B). The frequencies of these RR bands as well as their  $^{18}\text{O}$  isotopic sensitivities are consistent with  $\nu(\text{Fe-O})$  and  $\nu(\text{O-O})$  assignments of an  $\text{Fe}^{\text{III}}\text{-(hydro)peroxo}$  species, as described previously for the E47A<sup>[3b]</sup> and E114A<sup>[3c]</sup> mutants. Similarly to the I118S mutant (Figure 2A), bands in the 300  $\text{cm}^{-1}$  region and at 742  $\text{cm}^{-1}$  can be assigned to vibrational modes of the  $\text{Fe}^{\text{III}}\text{-(S-C(Cys))}$  group. The band at 867  $\text{cm}^{-1}$ , which was downshifted to 821  $\text{cm}^{-1}$  (-46) after mixing with  $\text{H}_2^{18}\text{O}_2$  (Figure 3B), could be attributed to  $\text{H}_2\text{O}_2$  weakly bound to the ferric iron,<sup>[11]</sup> taking into account that formation of this complex could be favoured by the large excess of hydrogen peroxide used with this mutant. Interestingly, an additional band at 826-828  $\text{cm}^{-1}$  was present (Figures 3A and 3B), which was not observed in the RR spectra recorded after reaction of the E47A<sup>[3b]</sup> and E114A<sup>[3c]</sup> mutants with  $\text{H}_2\text{O}_2$ . This 826-828  $\text{cm}^{-1}$  band appeared to be  $^{18}\text{O}$ -sensitive, possibly downshifting to 794  $\text{cm}^{-1}$  as a noticeable shoulder on the dominant 802  $\text{cm}^{-1}$  band (Figure 3B).

Longer incubation time of the K48I mutant with  $\text{H}_2^{16}\text{O}_2$  (30 sec) before freezing showed an increase in intensity of the 826  $\text{cm}^{-1}$  feature, compared to the 850, 742 and 444  $\text{cm}^{-1}$  bands (upper spectra Figures 3C and 3D). After mixing with  $\text{H}_2^{18}\text{O}_2$ , the band at 826  $\text{cm}^{-1}$  downshifted to 795  $\text{cm}^{-1}$  (-31) (lower spectra Figure 3D). Compared to the spectra obtained after 5 sec incubation time, no modification of the other RR bands was observed (Figures 3A and 3C). Similarly to the 826  $\text{cm}^{-1}$  line of the I118S mutant (Figure 2), the 826  $\text{cm}^{-1}$  band of the K48I mutant can be attributed to a  $\nu(\text{Fe=O})$  stretching mode of a high-valent iron-oxospecies. However, formation of the Fe=O species in the K48I mutant was slower, in agreement with the increase of the intensity of the 826  $\text{cm}^{-1}$  band from 5 to 30 sec incubation times (Figures 3B and 3D). Interestingly, the maximal formation of the  $\text{Fe}^{\text{III}}\text{-OOH}$  species in the K48I mutant occurred before that of the iron-oxo species. These data suggest that for the K48I mutant, the Fe=O species was formed from the  $\text{Fe}^{\text{III}}\text{-OOH}$  species. The fact that the intensities of the 444 and 850  $\text{cm}^{-1}$  RR bands remain constant from 5 to 30 sec incubation time (Figures 3A and 3C) could reflect a steady-state concentration of the  $\text{Fe}^{\text{III}}\text{-OOH}$  species, resulting from its formation and its conversion into the Fe=O species. These results are consistent with a mechanism involving a cleavage of the O-O bond of  $\text{Fe}^{\text{III}}\text{-OOH}$  to form the iron-oxo species, instead of a cleavage of the Fe-O bond in wild-type



**Figure 3.** Resonance Raman spectra of the K48I 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  $\text{H}_2\text{O}_2$ , rapidly mixed and frozen in liquid  $\text{N}_2$  after either 5 sec (A and B) or 30 sec (C and D) incubation time at 5 °C. A and C: summation of 8-9 scans; B and D: summation of 30 scans; The contribution of free  $\text{H}_2\text{O}_2$  was subtracted. Upper spectra, reaction with  $\text{H}_2^{16}\text{O}_2$ . Lower spectra, reaction with  $\text{H}_2^{18}\text{O}_2$ .

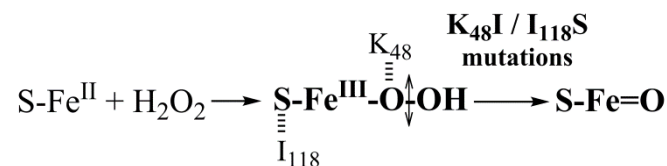
SOR to form  $\text{H}_2\text{O}_2$ .<sup>[2,3]</sup> These data are in line with the X-ray structure of the SOR  $\text{Fe}^{\text{III}}\text{-OOH}$  intermediates,<sup>[3a]</sup> which suggested

that the K48 residue could be involved in a specific protonation of the proximal oxygen of the intermediate, leading to the Fe-O bond cleavage. In the K48I mutant, such a specific protonation process should be canceled, and therefore could favour an O-O bond cleavage of the  $\text{Fe}^{\text{III}}\text{-OOH}$  intermediate.<sup>[5a]</sup>

For the I118S mutant, formation of the iron-oxo species could also result from the cleavage of the O-O bond of an  $\text{Fe}^{\text{III}}\text{-OOH}$  intermediate. Nevertheless, the faster rate of formation of the iron-oxospecies observed here for this mutant could explain the absence of RR signal of the  $\text{Fe}^{\text{III}}\text{-OOH}$  species (Figure 2). The I118S mutation was recently shown to impair a H-bond between the I118 NH peptide main chain and the sulfur atom of the cysteine ligand (Figure 1), leading to a strengthening of the S-Fe bond.<sup>[5b]</sup> It was shown that when the I118S mutant was reacted with  $\text{O}_2^{\bullet-}$ , the strengthening of the S-Fe bond induced an increase of the  $\text{p}K_a$  of the first reaction intermediate, proposed as being a ferrous iron superoxo species.<sup>[5a,b]</sup> Similarly to the observation made for the ferrous iron superoxo intermediate, it is conceivable that the I118S mutation could also modify the  $\text{p}K_a$  of the  $\text{Fe}^{\text{III}}\text{-OOH}$  species formed from the reaction with  $\text{H}_2\text{O}_2$ . Such a  $\text{p}K_a$  modification might favour protonation of the distal oxygen of the  $\text{Fe}^{\text{III}}\text{-OOH}$  species, leading to the cleavage of the O-O bond and formation of an Fe=O species in the I118S mutant. Indeed, conversion of a high-spin TMC  $\text{Fe}^{\text{III}}\text{-OOH}$  complex into iron-oxo species via O-O bond cleavage was recently shown to be facilitated by a protonation on the distal oxygen position.<sup>[12,13]</sup>

Alternatively, it is possible that in the I118S mutant the absence of a H-bond on the thiolate ligand and its consequent increase of the electronic density on the iron atom<sup>[5b]</sup> could allow the stabilisation of a high-valent iron-oxo species in the active site. Therefore in this mutant, formation of an Fe=O species may be favoured compared to the wild-type protein. Further studies, in particular DFT QM/MM calculations, should be required to specify the mechanism by which the I118S mutation leads to the formation of an Fe=O species.

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



**Figure 4.** Formation of Fe=O species in the I118S and K48I SOR mutants.

In summary, we report the first direct evidence that the non-heme mononuclear  $[\text{FeN}_4\text{S}_1]$  site of SOR can accommodate a high-valent iron-oxo species (Figure 4). Up to now, the roles of second coordinating sphere positions in oxygen activation processes have been hardly documented on synthetic iron complexes and on cytochrome P450, where for the latter direct characterization of one of its reaction intermediates, compound I, has been obtained only very recently.<sup>[1d]</sup> This work illustrates the fact that SOR may account for 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



SORmutants can specifically generate either  $\text{Fe}^{\text{III}}\text{-OOH}$  or  $\text{Fe}=\text{O}$  species upon reaction with  $\text{H}_2\text{O}_2$  provides an unprecedented tool to investigate the reactivity of each of these species in non-hememetalloenzymes. The oxidation properties of the different SOR mutants are currently under investigation.

**Keywords:** bioinorganic chemistry • non-heme iron-oxo intermediate • iron hydroperoxo intermediate • raman spectroscopy • superoxide reductase

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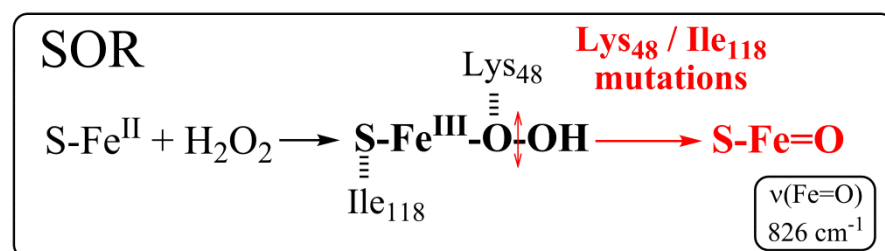
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- [13] That a homolytic or a heterolytic cleavage of the O-O bond of the  $\text{Fe}^{\text{III}}\text{-OOH}$  species occurs in SOR to generate an  $\text{Fe}^{\text{IV}}=\text{O}$  or an  $\text{Fe}^{\text{V}}=\text{O}$  species, respectively, cannot be deduced from RR experiments. In the sole example of an  $\text{Fe}^{\text{V}}=\text{O}$  unit characterized to date by RR (K. M. Van Heuvelen, A. T. Fiedler, X. Shan, R. F. De Hont, K. K. Meier, E. L. Bominaar, E. Münck, L. Que Jr., *Proc. Natl. Acad. Sci. U.S.A.*, **2012**, *109*, 11933-11938), the  $\nu(\text{Fe}-\text{O})$  frequencies (798-811  $\text{cm}^{-1}$ ) fall at the low-frequency end of the range observed for  $\text{Fe}^{\text{IV}}=\text{O}$  counterpart complexes.

## Table of Contents

### Iron-Oxo Species in SOR

Florence Bonnot, Emilie Tremey, David von Stetten, Stéphanie Rat, Simon Duval, Philippe Carpentier, Martin Clemancey, Alain Desbois, Vincent Nivière\*

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



Superoxide Reductase (SOR) as a model to study the mechanisms of formation of high-valent iron-oxo species in metalloenzymes. The second coordination sphere residues Lys48 and Ile118 tightly control the evolution and the cleavage of the O-O bond of the ferric iron hydroperoxide intermediate formed in the SOR active site.

# Supporting Information

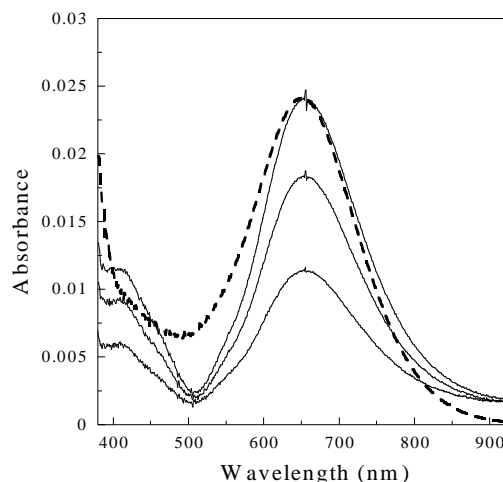
## 1. Materials and Methods

**Materials.**  $\text{H}_2^{18}\text{O}_2$  (90%  $^{18}\text{O}$ -enriched minimum) was purchased from ICON Services Inc. (Summit, NJ, USA).

**Protein purifications.** Over-expressions and purifications of the K48I and I118S SOR proteins from *Desulfoarcusbaarsii* were carried out as reported previously.<sup>[1,2]</sup> In the purified proteins, center I, the additional rubredoxin-distorted iron center present in the SOR from *Desulfoarcusbaarsii* and located at 22 Å from the iron active site,<sup>[3]</sup> was in a ferric state ( $\epsilon_{503\text{nm}} = 4.4 \text{ mM}^{-1} \text{ cm}^{-1}$ ). The iron active state was isolated in an oxygen-stable ferrous state.<sup>[1,2]</sup> The ferric iron active state, obtained after oxidation of the ferrous form with 2 equivalents of  $\text{Ir}^{\text{IV}}\text{Cl}_6$ , exhibited a broad absorption band with a maximum around 650 nm (K48I SOR mutant,  $\epsilon_{644\text{nm}} = 2.3 \text{ mM}^{-1} \text{ cm}^{-1}$ , pH 6.0; I118S SOR mutant,  $\epsilon_{651\text{nm}} = 1.9 \text{ mM}^{-1} \text{ cm}^{-1}$ , pH 8.5).

**Resonance Raman spectroscopy.** For the resonance Raman (RR) experiments, 3.5  $\mu\text{L}$  of the concentrated protein (4-6 mM) were deposited on a glass slide sample holder and then transferred into a cold helium gas circulating optical cryostat (STVP-100, Janis Research), held at 15 K. RR spectra were recorded using a Jobin-Yvon U1000 spectrometer, equipped with a liquid nitrogen-cooled CCD detector (Spectrum One, Jobin-Yvon, France). Excitation at 647.1 nm (30 mW) was provided by an Innova  $\text{Kr}^+$  laser (Coherent, Palo Alto). The signal-to-noise ratios were improved by spectral collections of 6 cycles of 30 s accumulation time. The spectrometer calibration was done as previously described.<sup>[4]</sup> The frequencies of the resonance Raman bands of SORs were also internally calibrated against the main band of the ice lattice ( $230 \text{ cm}^{-1}$ ) and a residual laser emission at 676.4 nm ( $669 \text{ cm}^{-1}$ ). In the spectra, the RR contribution of the SOR center I (the rubredoxin-like iron center) was subtracted using its marker band at  $382 \text{ cm}^{-1}$ .<sup>[5]</sup> In the  $200\text{-}320 \text{ cm}^{-1}$  region, the Raman signals of the ice were also removed. The intensities of the final spectra were scaled using the  $\nu(\text{CS})$  mode at  $742 \text{ cm}^{-1}$ . All these spectral treatments as well as the spectral analysis were made using the Grams 32 software (Galactic Industries). The frequency precision was  $0.5\text{-}1 \text{ cm}^{-1}$  for the most intense bands and  $1.5\text{-}2 \text{ cm}^{-1}$  for the weakest bands. Preliminary RR experiments were carried out using the microspectrophotometer setup at the Cryobench laboratory at the ESRF (Grenoble, France).<sup>[6]</sup> Cryo-loops were dipped in concentrated protein solutions and flash-cooled in a nitrogen cryo-stream at 100 K. RR spectra were recorded using a Renishaw InVia spectrophotometer with a 633 nm excitation (HeNe laser, 10 mW) and a RenCam CCD detector.

## 2. Figure S1



**Figure S1.** UV-visible absorption spectra of the I118S SOR mutant from *D. baarsii* (100  $\mu$ M in 10 mMTris/HCl pH 8.5) recorded, from the bottom to the top, 15, 40 and 80 sec after reaction with 1 molar equivalent  $\text{H}_2\text{O}_2$ , at room temperature (solid lines). The dashed line shows the spectrum of the I118S SOR mutant (13  $\mu$ M, 10 mMTris/HCl pH 8.5) oxidized with 2 molar equivalents of  $\text{Ir}^{\text{IV}}\text{Cl}_6$ . The contribution of the ferric iron center I (the additional rubredoxin-distorted iron center present in the SOR from *Desulfoarculusbaarsii*) recorded before  $\text{H}_2\text{O}_2$  or  $\text{Ir}^{\text{IV}}\text{Cl}_6$  treatments was subtracted in each spectrum.

As shown in Figure S1, a weak absorption band centered at 650 nm was rapidly formed upon addition of  $\text{H}_2\text{O}_2$  to the I118S SOR mutant, with a shape resembling that of the SOR ferric iron active site.<sup>[2]</sup> However, comparison of these spectra with one of a 13  $\mu$ M solution of SOR oxidized with  $\text{Ir}^{\text{IV}}\text{Cl}_6$  (dashed line in Figure S1), which exhibited the same absorbance value at 650 nm than the spectrum recorded 80 sec after mixing with  $\text{H}_2\text{O}_2$ , showed the presence of a residual absorption in the 750-950 nm region. This weak additional near-IR absorption was found to be reproducible and could not be associated to a difference in baseline.

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