Iron–Oxo Species | Hot Paper

DOI: 10.1002/ange.201400356

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: *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 ($O_2^{\bullet-}$) detoxification in microorganisms,[2] presents at least two striking similarities to cytochrome P450 oxygenase (Figure 1).[1b,c]

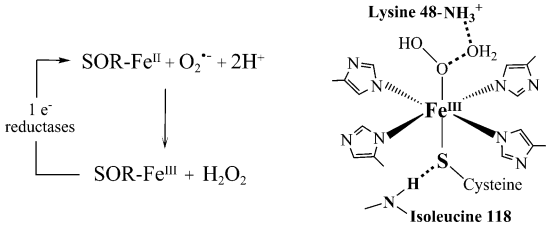

Figure 1. SOR detoxification activity and schematic representation of its ferric iron active site bound to a peroxide, as determined from its crystal structure (PDB ID: 2ji3).[3a] Hydrogen bonds are shown as dotted lines.

First, both enzymes contain a mononuclear iron site with an $\{FeN_4S_1\}$ 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

[*] Dr. F. Bonnot, Dr. E. Tremey, Dr. S. Rat, Dr. S. Duval, Dr. M. Clemancey, Dr. V. Nivière
Univ. Grenoble Alpes, iRTSV-LCBM, 38000 Grenoble (France)
and
CNRS, IRTSV-LCBM, 8000 Grenoble (France)
and
CEA, iRTSV-LCBM, 38000 Grenoble (France)
E-mail: vniviere@cea.fr
Dr. D. von Stetten, Dr. P. Carpentier
Structural Biology Group, European Synchrotron Radiation Facility
38043 Grenoble (France)
Dr. A. Desbois
Laboratoire Stress Oxydant et Détoxication
SB2SM and UMR 8221 CNRS-CEA-Université Paris Sud
iBiTec-S, CEA Saclay
91191 Gif-sur-Yvette Cedex (France)

[**] We are grateful to the French National Agency for Research (ANR) "Programme Labex" (ARCANE project ANR-11-LABX-003) for funding. Dr. Stéphane Ménage and Dr. Jean-Marc Latour are acknowledged for fruitful discussions.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201400356.

evolution of the Fe^{III}–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 Fe^{III}–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, K48 and I118.

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 Fe^{III}–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 Fe^{III}–S(Cys) moiety and of the internal cysteine ligand, respectively (Figure 2 A).[6] Accordingly, these RR bands were not sensitive to isotopic labeling after reaction with $H_2{}^{18}O_2$ (Figure 2 A).

Two other bands were observed at 826 and 845 cm^{-1} (Figure 2 A and B). These two bands could not be attributed to a ν(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 ν(Fe–O) stretching mode was detected in the 440–450 cm^{-1} region (Figure 2 A). The origin of the minor band at 845 cm^{-1} is difficult to assign as its isotopic sensitivity was not clearly seen (Figure 2 B). On the contrary, the dominant band at 826 cm^{-1} exhibited a shift by −33 cm^{-1} upon $^{16}O/^{18}O$ substitution (793 cm^{-1}; Figure 2 A and B). This value is much lower than that expected for a pure ν(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 ν(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 ν(Fe=O) modes in the 810–860 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 [Fe^{II}(TMC)(NCS)] complex was shown to form an Fe^{IV}=O species with a ν(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 ($\varepsilon = 100$–$400\ M^{-1}cm^{-1}$).[1e,f] As shown in Figure S1, the absorbance spectrum that results from the reaction of the

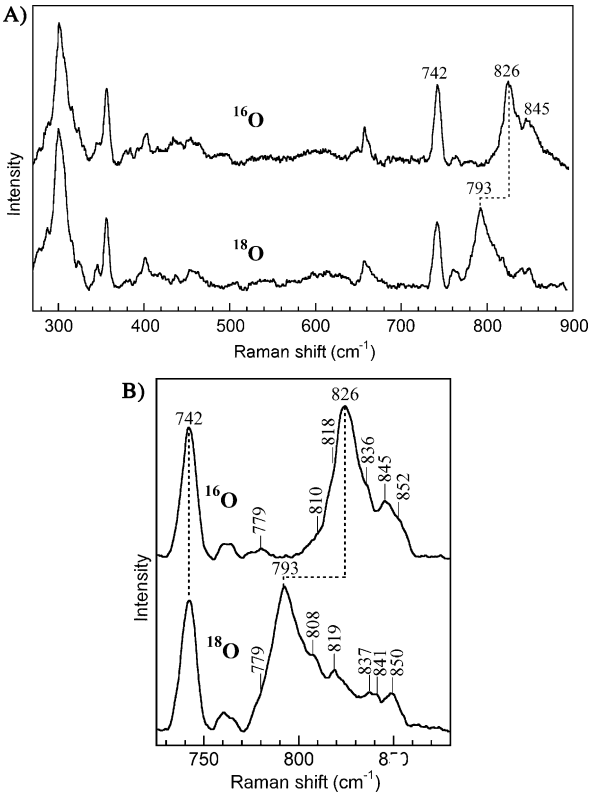

Figure 2. Resonance Raman spectra of the $I_{11}8S$ SOR mutant from *D. baarsii* (4 mM in 50 mM Tris/HCl, pH 8.5) excited at 647.1 nm (30 mW) at 15 K. The SOR solution (ferrous form) was treated with four equivalents of H_2O_2, rapidly mixed, and frozen in liquid N_2 after an incubation time of five 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 $H_2{}^{16}O_2$; lower spectra: reaction with $H_2{}^{18}O_2$.

$I_{118}S$ 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 Fe^{III}–S(Cys) moiety in the 300 cm^{-1} region of the RR spectra (Figure 2 A). However, a comparison of this absorbance spectrum with that of an $I_{118}S$ SOR mutant that was oxidized with $Ir^{IV}Cl_6$ (which only generates the ferric iron form) revealed the formation of additional bands in the 750–950 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 $H_2{}^{18}O_2$ (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 ν(Fe–O) and ν(O–O) assignments for an iron(III)–(hydro)peroxo species, as described previously for the $E_{47}A$[3b] and $E_{114}A$[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 Fe^{III}–(S–C(Cys)) group. The band at 867 cm^{-1}, which was downshifted to 821 cm^{-1} (−46 cm^{-1}) after mixing with $H_2{}^{18}O_2$ (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 $E_{47}A$[3b] and $E_{114}A$[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 $H_2{}^{16}O_2$ (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 $H_2{}^{18}O_2$, 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 ν(Fe=O) stretching mode of a high-valent iron–oxo species. However, formation of the Fe=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 Fe^{III}–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 Fe=O species was formed from the Fe^{III}–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 Fe^{III}–OOH species, resulting from its formation and subsequent conversion into the Fe=O species. These results are consistent with a mechanism that involves cleavage of the O–O bond of the Fe^{III}–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 Fe^{III}–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 Fe^{III}–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

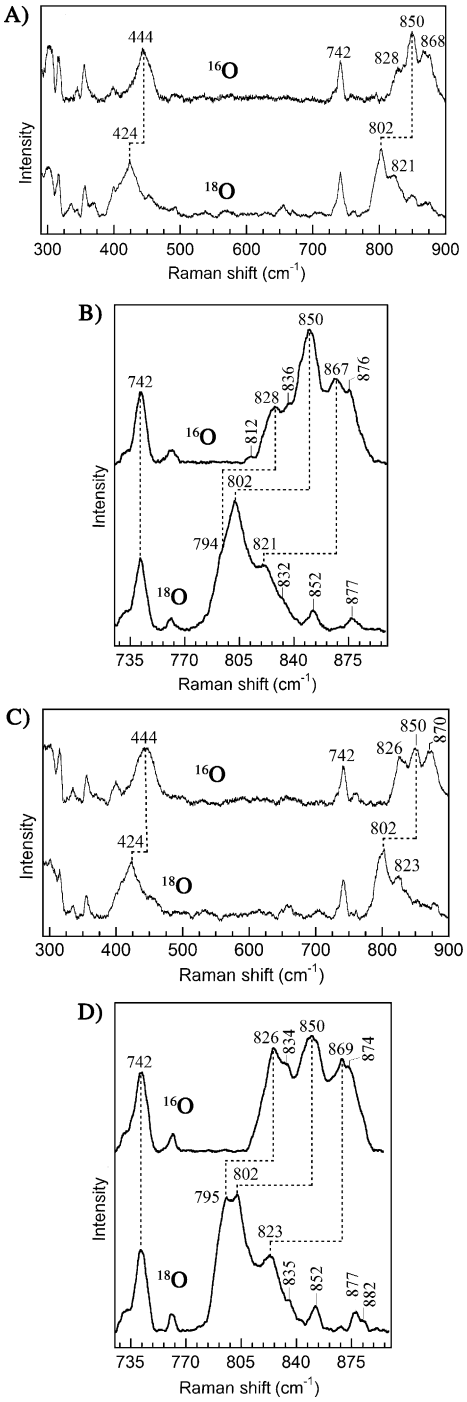

Figure 3. Resonance Raman spectra of the $K_{48}I$ SOR mutant from *D. baarsii* (4 mM in 50 mM Tris/HCl, pH 8.5) excited at 647.1 nm (30 mW) at 15 K. The SOR solution (ferrous form) was treated with 30 equivalents of H_2O_2, rapidly mixed, and frozen in liquid N_2 after incubation times of either 5 s (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 $H_2{}^{16}O_2$; lower spectra: reaction with $H_2{}^{18}O_2$.

Fe^{III}–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 Fe^{III}–OOH species (Figure 2). The $I_{118}S$ mutation was

recently shown to impair a 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), which leads to a strengthening of the S–Fe bond.[5b] When the $I_{118}S$ mutant reacted with $O_2^{\bullet-}$, 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 $I_{118}S$ mutation could also modify the pK_a value of the Fe^{III}–OOH species that is formed by the reaction with H_2O_2. 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 $I_{118}S$ 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 $I_{118}S$ 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 $I_{118}S$ 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 K_{48} and I_{118} (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.

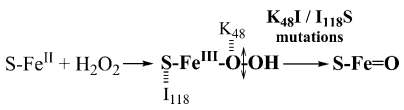

Figure 4. The formation of Fe=O species in the $I_{118}S$ and $K_{48}I$ SOR mutants.

In summary, we have reported the first direct evidence that the non-heme mononuclear $\{FeN_4S_1\}$ 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 H_2O_2 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.

Received: January 13, 2014
Revised: March 13, 2014
Published online: April 28, 2014

Keywords: bioinorganic chemistry · iron · metalloenzymes · Raman spectroscopy · superoxide reductase

[1] a) M. Costas, M. P. Mehn, M. P. Jensen, L. Que, Jr., *Chem. Rev.* **2004**, *104*, 939–986; b) I. G. Denisov, T. M. Makris, S. G. Sligar, I. Schlichting, *Chem. Rev.* **2005**, *105*, 2253–2277; c) S. Shaik, S. Cohen, Y. Wang, H. Chen, D. Kumar, W. Thiel, *Chem. Rev.* **2010**, *110*, 949–1017; d) J. Rittle, M. T. Green, *Science* **2010**, *330*, 933–937; e) A. R. McDonald, L. Que, Jr., *Coord. Chem. Rev.* **2013**, *257*, 414–428; f) S. P. de Visser, J. U. Rohde, Y. M. Lee, J. Cho, W. Nam, *Coord. Chem. Rev.* **2013**, *257*, 381–393.

[2] a) F. E. Jenney, Jr., M. F. J. M. Verhagen, X. Cui, M. W. W. Adams, *Science* **1999**, *286*, 306–309; b) M. Lombard, M. Fontecave, D. Touati, V. Nivière, *J. Biol. Chem.* **2000**, *275*, 115–121; c) D. M. Kurtz, Jr., *Acc. Chem. Res.* **2004**, *37*, 902–908; d) A. S. Pereira, P. Tavares, F. Folgosa, R. M. Almeida, I. Moura, J. J. G. Moura, *Eur. J. Inorg. Chem.* **2007**, 2569–2581; e) A. F. Pinto, J. V. Rodrigues, M. Teixeira, *Biochim. Biophys. Acta Proteins Proteomics* **2010**, *1804*, 285–297; f) V. Niviere, F. Bonnot, D. Bourgeois in *Handbook of Metalloproteins*, *Vols. 4 & 5* (Ed.: A. Messerschmidt), Wiley, Chichester, **2011**, pp. 246–258.

[3] a) G. Katona, P. Carpentier, V. Nivière, P. Amara, V. Adam, J. Ohana, N. Tsanov, D. Bourgeois, *Science* **2007**, *316*, 449–453; b) C. Mathé, T. A. Mattioli, O. Horner, M. Lombard, J. M. Latour, M. Fontecave, V. Nivière, *J. Am. Chem. Soc.* **2002**, *124*, 4966–4967; c) C. Mathé, C. O. Weill, T. A. Mattioli, C. Berthomieu, C. Houée-Levin, E. Tremey, V. Nivière, *J. Biol. Chem.* **2007**, *282*, 22207–22216.

[4] a) L. M. Brines, J. A. Kovacs, *Eur. J. Inorg. Chem.* **2007**, 29–38; b) M. R. Bukowski, H. L. Halfen, T. A. van den Berg, J. A. Halfen, L. Que, Jr., *Angew. Chem.* **2005**, *117*, 590–593; *Angew. Chem. Int. Ed.* **2005**, *44*, 584–587.

[5] a) F. Bonnot, T. Molle, S. Ménage, Y. Moreau, S. Duval, V. Favaudon, C. Houée-Levin, V. Nivière, *J. Am. Chem. Soc.* **2012**, *134*, 5120–5130; b) E. Tremey, F. Bonnot, Y. Moreau, C. Berthomieu, A. Desbois, V. Favaudon, G. Blondin, C. Houée-Levin, V. Nivière, *J. Biol. Inorg. Chem.* **2013**, *18*, 815–830.

[6] a) M. D. Clay, J. P. Emerson, E. D. Coulter, D. M. Kurtz Jr., M. K. Johnson, *J. Biol. Inorg. Chem.* **2003**, *8*, 671–682; b) M. D. Clay, F. E. Jenney, Jr., H. J. Noh, P. L. Hagedoorn, M. W. W. Adams, M. K. Johnson, *Biochemistry* **2002**, *41*, 9833–9841.

[7] P. K. Grzyska, E. H. Appelman, R. P. Hausinger, D. A. Proshlyakov, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 3982–3987.

[8] A. Desbois, M. Momenteau, M. Lutz, *Inorg. Chem.* **1989**, *28*, 825–834.

[9] The band at 826 cm^{-1} in the $I_{118}S$ SOR mutant was not shifted in the presence of $H_2{}^{18}O$ (data not shown), which suggests that the oxygen atom of the Fe=O species is not exchangeable with water. For non-heme iron complexes, the mechanism of Fe=O oxygen exchange with water was proposed to involve an available coordination position in a *cis* configuration with respect to the oxo group; see: A. Company, I. Prat, J. R. Frisch, R. Mas Ballesté, M. Güell, G. Juhász, X. Ribas, E.

Münck, J. M. Luis, L. Que, Jr., M. Costas, *Chem. Eur. J.* **2011**, *17*, 1622 – 1634. Such available positions are lacking in the SOR iron site,[3a] which could explain the absence of $H_2{}^{18}O$ sensitivity of the Fe=O species.

[10] C. V. Sastri, M. J. Park, T. Ohta, T. A. Jackson, A. Stubna, M. S. Seo, J. Lee, J. Kim, T. Kitagawa, E. Münck, L. Que, Jr., W. Nam, *J. Am. Chem. Soc.* **2005**, *127*, 12494 – 12495.

[11] An $Fe^{III}-H_2O_2$ complex was proposed as a late reaction intermediate during the reaction of SOR with superoxide; see: A. Dey, F. E. Jenney, Jr., M. W. Adams, M. K. Johnson, K. O. Hodgson, B. Hedman, E. I. Solomon, *J. Am. Chem. Soc.* **2007**, *129*, 12418 – 12431.

[12] F. Li, K. K. Meier, M. A. Cranswick, M. Chakrabarti, K. M. Van Heuvelen, E. Munck, L. Que, Jr., *J. Am. Chem. Soc.* **2011**, *133*, 7256 – 7259.

[13] It cannot be deduced from RR experiments whether a homolytic or a heterolytic cleavage of the O–O bond of the Fe^{III}–OOH species occurred in SOR to generate an Fe^{IV}=O or an Fe^{V}=O species, respectively. In the sole example of an Fe^{V}=O unit that was characterized to date by RR, the ν(Fe–O) frequencies (798 – 811 cm^{-1}) fall at the low-frequency end of the range observed for the Fe^{IV}=O counterpart complexes; see: 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. USA* **2012**, *109*, 11933 – 11938.