Identification of Iron (III) Peroxo Species in the Active Site of the Superoxide Reductase SOR from *Desulfoarculus baarsii*.

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Superoxide reductase (SOR) is a newly discovered activity by which some anaerobic or microaerophilic organisms eliminate superoxide, O$_2^-$.[^1] The SOR catalyzed reaction differs from that of well-known superoxide dismutases SOD in that it does not produce O$_2$, but instead reduces by one electron O$_2$ to form H$_2$O$_2$ exclusively: O$_2^- + 1 e^- + 2H^+ \rightarrow H_2O_2$.

The active site of SOR consists of a Fe$^{2+}$ center (center II) in an unusual [His$_x$ Cys$_y$] square pyramidal pentacoordination.[^2] It reacts specifically at a nearly diffusion-controlled rate with O$_2^-$, generating H$_2$O$_2$ and the oxidized form of the enzyme, the ferric iron center II. The SORs (originally called desulfoferrodoxin) found in some sulfate reducing bacteria, e.g. *Desulfovibrio desulfuricans*[^2a,3] or *Desulfoarculus baarsii*,[^1b] contain an additional mononuclear Fe$^{3+}$ center, called center I, coordinated by four cysteines with a distorted rubredoxin-type structure. However, center I is not required for the reaction and, up to now, its function remains unknown.[^1b,c]

Recent pulse radiolysis studies of the reaction of center II with O$_2^-$ have allowed the observation, in the micro and millisecond time scale, of intermediates characterized by absorption bands in the 550-650 nm range.[^3] These transient species were proposed to be Fe$^{3+}$ peroxy complexes, from which H$_2$O$_2$ is liberated, on the assumption of an inner sphere mechanism. The absorption band at 560 nm resulted probably mainly from the Cys-to-Fe$^{3+}$ charge transfer band,[^3] but also the 4.2 K EPR spectrum,[^7] after subtraction of signals from center I, recorded just after addition of 6 equivalents of H$_2$O$_2$, was complex, with a major feature at g = 4.3 and a minor one at g = 4.15 (Fig.1Bi). The former one is comparable to that of an EPR spectrum of SOR E47A oxidized with hexachloroiridate (IV) (Fig.1Bii). It is characteristic for a high-spin Fe$^{3+}$ in a rhombic ligand field.[^1b] No other signals in the g = 2 and g = 8-10 regions were observed. At longer incubation time (10 min) with H$_2$O$_2$, the feature at g = 4.15 completely disappeared (data not shown).

When the reaction was carried out with the wild-type SOR and H$_2$O$_2$, under the same conditions that we described above for the mutant, an intense RR band at 743 cm$^{-1}$ was observed (Fig. 2a). This band can be used as a marker of the amount of Fe$^{3+}$ formed in these conditions. The 4.2 K EPR spectra of the SOR wild-type, after addition of H$_2$O$_2$, exhibited the rhombic signal at g = 4.3.[^1b,3] whereas the feature at g = 4.15 was very weak and completely vanished within a few min (data not shown).

The observed RR frequencies at 850 and 438 cm$^{-1}$ and their $^{18}$O isotopic shifts (-48 and -23 cm$^{-1}$) are consistent with the v(Fe-O$_2$) and v(O-O) stretching modes, respectively, of an Fe$^{3+}$-peroxo species.[^9] The lack of deuterium isotopic shifts suggests that this peroxo species is not protonated. We thus conclude that H$_2$O$_2$ can oxidize SOR and bind to the ferric center II to yield a transient high-spin Fe$^{3+}$-peroxo species, associated with the feature at g = 4.15, as observed from the 4.2 K EPR spectra. The absorption band at 560 nm resulted probably mainly from the Cys-to-Fe$^{3+}$ charge transfer band,[^3] but also...
contains a contribution of the peroxo-to-iron Fe\(^{3+}\) charge transfer band.\(^9\) The resolution of these two charge transfer bands could be achieved by a RR excitation profile, but this is complicated because of the strong interference of center I when excitations are made below 647 nm.\(^3\)

The observed Raman frequencies are comparable to those described for the end-on high-spin Fe\(^{3+}\)-OOH species in oxyhemerythrin which showed deuterium isotope shifts.\(^10\) However, for SOR reported here, the unusually low FeO\(_2\) frequency (438 cm\(^{-1}\)) strongly suggests a side-on \(\eta^2\) Fe\(^{3+}\)-peroxo species\(^1\) as found in the high-spin Fe complexes such as [(EDTA)Fe(\(\eta^2\)-O\(_2\))\(^2\)], for example.\(^2\) In addition, the lack of deuteron shift, suggesting a non-protonated peroxo species, is also consistent with a side-on \(\eta^2\) Fe\(^{3+}\)-peroxo species since it is expected to be more stable in the unprotonated form. Such a coordination in the SOR active site would thus imply either a heptacoordination for the iron or a loss of one of the imidazole ligands, but up to now there is no evidence for such possible coordination changes.\(^3\) Clearly, relevant model Fe-peroxo species with sulfur ligands, not yet available, would support our proposal of a side-on peroxo coordination in SOR.

In conclusion, the data presented here first show that SOR active site can accommodate a Fe\(^{3+}\)-peroxo species and thus support the hypothesis that reduction of O\(_2\) proceeds through such intermediates. To our knowledge, this is the first Fe\(^{3+}\)-(hydro)peroxo species that has been identified in a mononuclear non-heme iron protein, with such an unusual active site. Current RR experiments in the laboratory are directed in order to identify Fe\(^{3+}\)-peroxo species formed immediately after reaction with O\(_2\).

Second, the results suggest that the conserved Glu47 might serve to help H\(_2\)O\(_2\) release, as illustrated in Scheme 1, since mutation of that residue to alanine results in stabilization of the active site. Current RR experiments in the laboratory are directed in order to identify Fe\(^{3+}\)-peroxo species formed immediately after reaction with O\(_2\).

Figure 1. UV-visible (A) and X-band EPR spectra (B) of SOR E47A mutant from \(D.\) baarsii (200 \(\mu\)M in 50 mM Tris/ HCl pH 7.6) treated with 6 equivalents H\(_2\)O\(_2\) or 3 equivalents K\(_2\)IrCl\(_6\). (A) UV-Visible spectrum recorded 5 s after addition of H\(_2\)O\(_2\). (B) EPR spectrum after treatment with H\(_2\)O\(_2\) and immediately freezing after mixing, K\(_2\)IrCl\(_6\). The contribution of the high-spin Fe\(^{3+}\) center 1 [Fe(SCys)\(_4\)] was subtracted from each UV-visible and EPR spectrum. EPR conditions: temperature 4.2 K, microwave frequency 9.676 GHz, power 20 mW, modulation 1.0 mT/100 kHz.

Figure 2. Resonance Raman spectra of SOR E47A mutant and wild-type forms from \(D.\) baarsii (1 mM in 50 mM Tris/HCl pH 7.6) excited at 647.1 nm (50 mW) at 15 K. a): SOR E47A treated with 3 equivalents K\(_2\)IrCl\(_6\). b): SOR E47A treated with 6 equivalents of H\(_2\)O\(_2\) and immediately frozen (less than 5s). c): SOR E47A treated with H\(_{18}\)O\(_2\), same conditions as b). d): SOR wild-type treated with 6 equivalents of H\(_2\)O\(_2\) rapidly mixed and immediately frozen (less than 5s).

Supporting Information Available. Deuteron isotopic effects on the RR bands at 850 and 438 cm\(^{-1}\).

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(6) (a) The absorption spectrum of center II of the SOR E47A from \(D.\) baarsii oxidized with a slight molar excess of K\(_2\)IrCl\(_6\), is characterized by a band centered at 560 nm, \(\varepsilon = 1.6\) mM\(^{-1}\) cm\(^{-1}\). (b) At longer incubation times, the 560 nm absorption band rapidly shifts at 650 nm with a decrease in intensity, reflecting a possible degradation process due to excess of H\(_2\)O\(_2\).

(7) EPR spectra were recorded on a Bruker EMX spectrometer. For low-temperature studies, an Oxford Instrument continuous-flow helium cryostat and temperature control system were used.

(8) Resonance Raman spectra were recorded using instrumentation as reported in: Ollagnier-de-Choudens, S., Mattioli, T. A., Takahashi, Y., Fontecave, M. J. Biol. Chem. 2001, 276, 22564-22607. Final concentration of protein, held in a He gas circulating cryostat at 15 K, was 1 mM and 50 mW of 647.1 nm radiation from a Kr+ laser (Coherent Innovia 90) was used to excite the spectrum. Spectra were accumulated for 40 min and baselines were corrected using GRAMS 32 (Galactic Industries).

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The active site of superoxide reductase SOR consists of a Fe\(^{2+}\) center in an unusual [His\(_4\), Cys\(_1\)] square pyramidal geometry. It specifically reduces superoxide to produce H\(_2\)O\(_2\). Here, we have reacted the SOR from *Desulfoarculus baarsii* directly with H\(_2\)O\(_2\). We have found that its active site can transiently stabilize a Fe\(^{3+}\)-peroxo species that we have spectroscopically characterized by resonance Raman. The mutation of the strictly conserved Glu47 into alanine results in a stabilization of this Fe\(^{3+}\)-peroxo species, when compared to the wild-type form. These data support the hypothesis that the reaction of SOR proceeds through such Fe\(^{3+}\)-peroxo intermediate. This also suggests that Glu47 might serve to H\(_2\)O\(_2\) released during the reaction with superoxide.

**Figure S1.** Deuterium isotopic effects on the resonance Raman spectra for the ν(Fe-O\(_2\)) (left panel) and ν(O-O) (right panel) regions of SOR E47A mutant from *D. baarsii* (1 mM in 50 mM Tris/HCl pH 7.6, or pD 8.0) excited at 647.1 nm (50 mW) at 15 K, treated with 6 equivalents of H\(_2\)O\(_2\), rapidly mixed and immediately frozen (less than 5 s). Upper spectra in D\(_2\)O solution. Lower spectra in H\(_2\)O solution.
