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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, $O_2^{\cdot-}$.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 $O_2^{\cdot-}$ to form H_2O_2 exclusively: $O_2^{\cdot-} + 1 e^- + 2H^+ \rightarrow H_2O_2$.

The active site of SOR consists of a Fe^{2+} center (center II) in an unusual [$His_4 Cys_1$] square pyramidal pentacoordination.² It reacts specifically at a nearly diffusion-controlled rate with $O_2^{\cdot-}$, generating H_2O_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. *Desulfoarculus baarsii*^{1b} and *Desulfovibrio desulfuricans*,^{2a,3} 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 $O_2^{\cdot-}$ have allowed the observation, in the micro and millisecond time scale, of intermediates characterized by absorption bands in the 550-650 nm range.⁴ These transient species were proposed to be Fe^{3+} peroxo complexes, from which H_2O_2 is liberated, on the assumption of an inner sphere mechanism for $O_2^{\cdot-}$ reduction and on the basis that the corresponding absorption bands were slightly different from those of the final ferric iron center II.⁴

On the basis of the crystal structure^{2b} and spectroscopic studies⁵ of the SOR from *Pyrococcus furiosus*, it has been proposed that upon oxidation the iron active site becomes six-coordinated, as the consequence of a local protein domain movement which places a strictly conserved glutamate (Glu47 in the SOR from *D. baarsii*) in the free coordination site. We have mutated the Glu47 to alanine (E47A) in the SOR from *D. baarsii* and found that this mutation did not affect the kinetics of formation of the above mentioned intermediates detected by pulse radiolysis.^{4a-b} However, because this Glu residue becomes a ligand for the oxidized iron, a likely hypothesis could be that it serves to release H_2O_2 from the Fe^{3+} peroxo intermediate by substitution in the iron coordination sphere.

Here, we have reacted SOR E47A from *D. baarsii* directly with H_2O_2 and have found that the active site of the mutant can indeed transiently stabilize a Fe^{3+} peroxo species, that could be spectroscopically characterized.

When we rapidly manually mixed SOR E47A from *D. baarsii* with 6 equivalents of H_2O_2 , a UV-visible absorption feature with a maximum at 560 nm, characteristic for the oxidation of the iron center II,^{6a} was immediately observed (Fig.1A).^{6b} The

4.2 K EPR spectrum,⁷ after subtraction of signals from center I, recorded just after addition of 6 equivalents of H_2O_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,3} No other signals in the $g = 2$ and $g = 8-10$ regions were observed. At longer incubation time (10 min) with H_2O_2 , the feature at $g = 4.15$ completely disappeared (data not shown).

Resonance Raman (RR) spectra at 15 K,⁸ taken from the SOR E47A frozen immediately after addition of H_2O_2 indicated the presence of two new bands at 850 and 438 cm^{-1} (Fig.2b), which were not present when SOR was oxidized with hexachloroiridate (IV) (Fig.2a). The RR spectra also exhibit a band at 742 cm^{-1} which has been attributed to an internal C-S stretching mode of the $CysS-Fe^{3+}$ active site.³ When the same Raman measurements were made after mixing with $H_2^{18}O_2$, the 850 and 438 cm^{-1} bands were observed to down shift to 802 and 415 cm^{-1} , respectively (Fig.2c). RR measurements in D_2O buffer indicated no significant shifts of the 850 and 438 cm^{-1} bands to within 1 cm^{-1} (cf. Supporting Information).

When the reaction was carried out with the wild-type SOR and H_2O_2 , under the same conditions that we described above for the mutant, an intense RR band at 743 cm^{-1} was observed (Fig. 2d). This band can be used as a marker of the amount of Fe^{3+} formed in these conditions. The bands at 850 and 438 cm^{-1} observed in the case of the mutant with a similar amplitude as that of the 743 cm^{-1} band (Fig. 2b) were now in the case of the wild-type found to be very weak compared to the 743 cm^{-1} band (Fig. 2d). However, they exhibited the same shift upon ^{18}O substitution than reported in the case of the mutant (data not shown). The 4.2 K EPR spectra of the SOR wild-type, after subtraction of signal of center I, and recorded immediately after addition of H_2O_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 $\nu(O-O)$ and $\nu(Fe-O_2)$ 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_2O_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,^{3,5} 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 Fe-O_2 frequency (438 cm^{-1}) strongly suggests a side-on $\eta^2\text{Fe}^{3+}$ -peroxo species¹¹ as found in the high-spin Fe complexes such as $[(\text{EDTA})\text{Fe}(\eta^2\text{-O}_2)]^{3+}$, for example.⁹ In addition, the lack of deuterium shift, suggesting a non-protonated peroxo species, is also consistent with a side-on $\eta^2\text{Fe}^{3+}$ -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.⁵ 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 $\text{O}_2^{\cdot-}$ 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 $\text{O}_2^{\cdot-}$.

Second, the results suggest that the conserved Glu47 might serve to help H_2O_2 release, as illustrated in Scheme 1, since mutation of that residue to alanine results in stabilization of the Fe^{3+} peroxide. It should be noted that the presence of the cysteinate trans to the peroxide may also be crucial in promoting H_2O_2 dissociation from the Fe^{3+} -peroxo intermediate, by pushing electron density on the iron. As a matter of fact, the Fe-O_2 bond observed here, with $\nu = 438\text{ cm}^{-1}$, is particularly weak and the O-O bond with $\nu = 850\text{ cm}^{-1}$ strong, when compared to the corresponding values reported for model complexes that promote O-O cleavage and formation of high valent Fe-O species.⁹

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Supporting Information Available. Deuterium 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_2IrCl_6 is characterized by a band centered at 560 nm , $\epsilon = 1.6\text{ mM}^{-1}\text{ cm}^{-1}$. (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_2O_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*, 22604-22607. 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 Innova 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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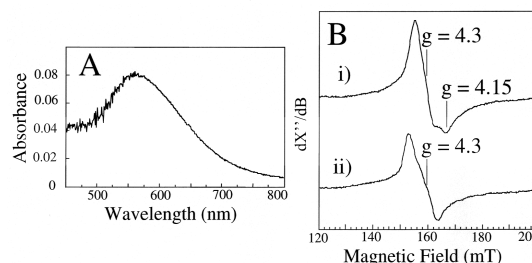


Figure 1. UV-visible (A) and X-band EPR spectra (B) of SOR E47A mutant from *D. baarsii* ($200\text{ }\mu\text{M}$ in 50 mM Tris/HCl pH 7.6) treated with 6 equivalents H_2O_2 or 3 equivalents K_2IrCl_6 . (A) UV-Visible spectrum recorded 5 s after addition of H_2O_2 . (B) EPR spectrum after treatment with i) H_2O_2 and immediate freezing after mixing, ii) K_2IrCl_6 . The contribution of the high-spin Fe^{3+} center I [$\text{Fe}(\text{SCys})_4$] was subtracted from each UV-visible and EPR spectrum. EPR conditions: temperature 4.2 K , microwave frequency 9.676 GHz , power 20 mW , modulation $1.0\text{ mT}/100\text{ kHz}$.

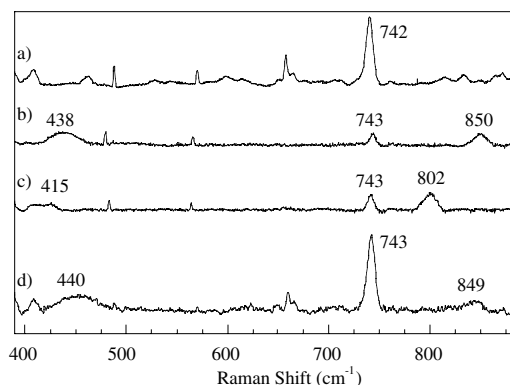
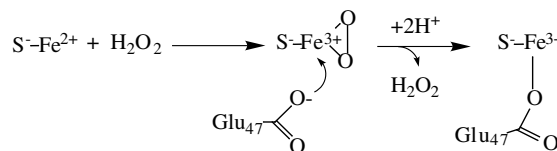


Figure 2. Resonance Raman spectra of SOR E47A mutant and wild-type forms from *D. baarsii* (1 mM in 50 mM Tris/HCl pH 7.6) excited at 647.1 nm (50 mW) at 15 K . a): SOR E47A treated with 3 equivalents K_2IrCl_6 . b): SOR E47A treated with 6 equivalents of H_2O_2 , rapidly mixed and immediately frozen (less than 5 s). c): SOR E47A treated with $\text{H}_2^{18}\text{O}_2$, same conditions as b). d): SOR wild-type treated with 6 equivalents of H_2O_2 rapidly mixed and immediately frozen (less than 5s).



Scheme 1.
