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Superoxide reductase (SOR) is a newly discovered activity by which some anaerobic or microaerophilic organisms eliminate superoxide, $O_2^{\bullet-}$.[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 $O_2^{\bullet-}$ to form H_2O_2 exclusively: $O_2^{\bullet-} + 1\ e^- + 2H^+ \rightarrow H_2O_2$.

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 $O_2^{\bullet-}$, 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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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 studies[5] 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 ν(O-O) and ν(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 η^2 Fe^{3+}-peroxo species[11] as found in the high-spin Fe complexes such as $[(EDTA)Fe(\eta^2\text{-}O_2)]^{3+}$, for example.[9] In addition, the lack of deuterium shift, suggesting a non-protonated peroxo species, is also consistent with a side-on η^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.[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 $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$.

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 ν = 438 cm^{-1}, is particular weak and the O-O bond with ν = 850 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}.

(1) (a) Jenney, F. E., Jr.; Verhagen, M. F. J. M.; Cui, X.; Adams, M. W. W. Science **1999**, *286*, 306-309. (b) Lombard, M.; Fontecave, M.; Touati, D.; Nivière, V. J. Biol. Chem. **2000**, *275*, 115-121. (c) Lombard, M.; Touati, D.; M., Fontecave; Nivière, V. J. Biol. Chem. **2000**, *275*, 27021-27026.

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(3) Tavares, P.; Ravi, N.; Moura, J. J. G.; LeGall, J.; Huang, Y. H.; Crouse, B. R.; Johnson, M. K.; Huynh, B. H.; Moura, I. J. Biol. Chem. **1994**, *269*, 10504-10510.

(4) (a) Lombard, M.; Houée-Levin, C.; Touati, D.; Fontecave, M.; Nivière, V. Biochemistry **2001**, *40*, 5032-5040. (b) The effect of the E47A mutation on the decay of the intermediates could not be investigated with the experimental procedure used in 4a (c) Coulter, E. D.; Emerson, J. P.; Kurtz, D. M.; Jr.; Cabelli, D. E. J. Am. Chem. Soc. **2000**, *122*, 11555-11556. (d) Nivière, V; Lombard, M.; Fontecave, M.; Houée-Levin, C. FEBS Letters **2001**, *497*, 171-173. (e) Abreu, I. A.; Saraiva, L. M.; Soares, C. M.; Teixeira, M.; Cabelli, D. E. J. Biol. Chem. **2001**, *276*, 38995-39001.

(5) Clay, M. D.; Jenney, F. E.; Hagedoorn, P. L.; George, G. N.; Adams, M. W. W.; Johnson, M. J. J. Am. Chem. Soc. **2002**, *124*, 788-813.

(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, ε = 1.6 mM^{-1} cm^{-1}. (b) At longer incubation times, the 560 nm absorption band rapidely 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).

(9) Girerd, J. J.; Banse, F.; Simaan, A., J. Structure and Bonding **2000**, *97*, 145-177, and references herein.

(10) Stenkamp, R. E. Chem. Rev. **1994**, *94*, 715-726, and references herein.

(11) A high-spin mononuclear Fe^{3+}-OOH complex displays a much stronger Fe-O_2 bond: Wada, A., Ogo, S., Nagatomo, S., Kitagawa, T., Watanabe, Y., Jitsukawa, K., Masuda, H. Inorg. Chem., **2002**, *41*, 616-618.

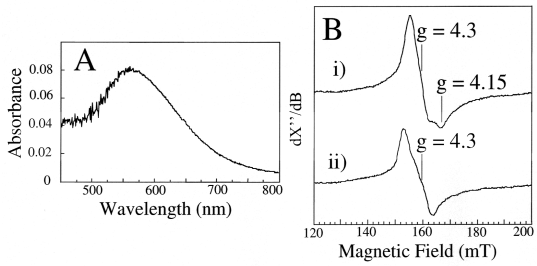


Figure 1. UV-visible (A) and X-band EPR spectra (B) of SOR E47A mutant from *D. baarsii* (200 µM in 50 mM Tris/HCl pH 7.6) treated with 6 equivalents H_2O_2 or 3 equivalents K_2IrCl_6. (A) UV-Visible spectrum recorded 5 s after addition of H_2O_2. (B) EPR spectrum after treatment with i) H_2O_2 and immediate freezing after mixing, ii) K_2IrCl_6. The contribution of the high-spin Fe^{3+} center I [$Fe(SCys)_4$] 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.

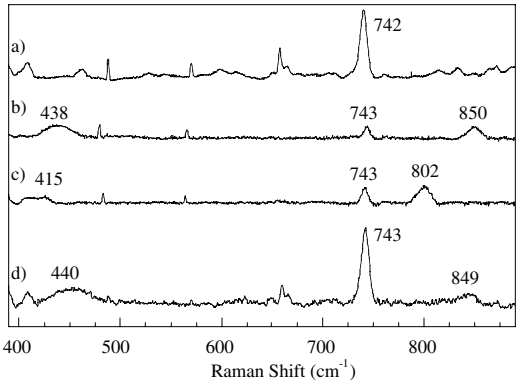
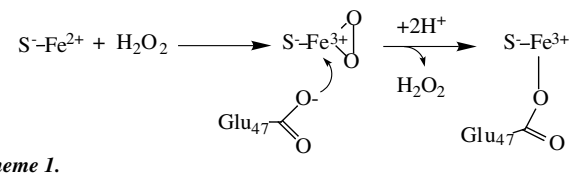
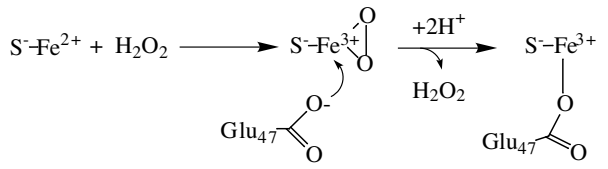


Figure 2. Resonance Raman spectra of SOR E47A mutant and wild-type forms from *D. baarsii* (1 mM in 50 mM Tris/HCl pH 7.6) excited at 647.1 nm (50 mW) at 15 K. a): SOR E47A treated with 3 equivalents K_2IrCl_6. b): SOR E47A treated with 6 equivalents of H_2O_2, rapidly mixed and immediately frozen (less than 5 s). c): SOR E47A treated with $H_2{}^{18}O_2$, 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.

Table of Contents graphic :



ABSTRACT FOR WEB PUBLICATION.

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_2O_2. Here, we have reacted the SOR from *Desulfoarculus baarsii* directly with H_2O_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_2O_2 released during the reaction with superoxide.

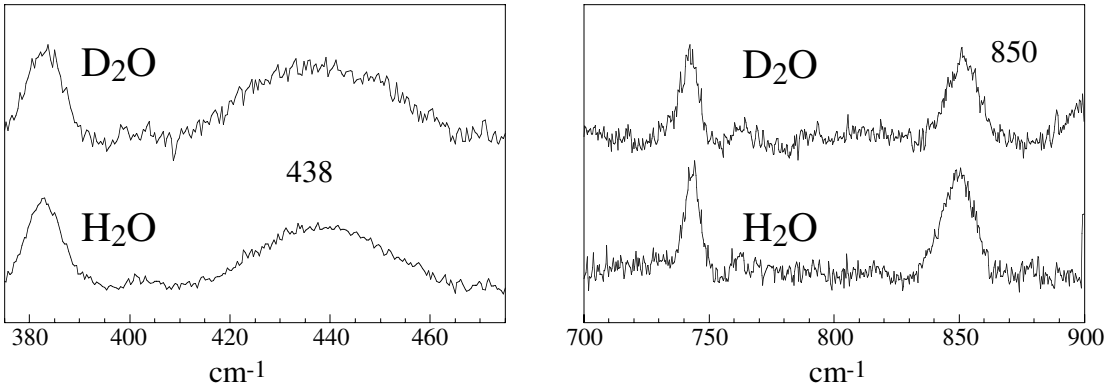


Figure S1. Deuterium isotopic effects on the resonance Raman spectra for the ν(Fe-O_2) (left panel) and ν(O-O) (right panel) regions of SOR E47A mutant from *D. baarsii* (1 mM in 50 mM Tris/HCl pH 7.6, or pD 8.0) excited at 647.1 nm (50 mW) at 15 K, treated with 6 equivalents of H_2O_2, rapidly mixed and immediately frozen (less than 5 s). Upper spectra in D_2O solution. Lower spectra in H_2O solution.