Non-heme iron hydroperoxo species in superoxide reductase as a catalyst of oxidation reactions

S. Rat,[a,b,c] S. Ménage[a,b,c] F. Thomas[d] and V. Nivière[a,b,c]*

[a] Univ. Grenoble Alpes, iRTSV-LCBM, F-38000 Grenoble, France
[b] CNRS, IRTSV-LCBM, F-38000 Grenoble, France
[c] CEA, iRTSV-LCBM, F-38000 Grenoble, France
[d] Département de Chimie Moléculaire – Chimie Inorganique Redox Biomimétique (CIRE) – UMR CNRS 5250, Univ. Grenoble Alpes, BP 53, 38041 Grenoble cedex 9, France
* E-mail: vniviere@cea.fr; Tel: 33 4 38 78 91 09

Electronic Supplementary Information

1) Materials and experimental procedures

Materials. $H_2{}^{18}O_2$ (90% ^{18}O-enriched) was purchased from ICON Services Inc. (Summit, NJ, USA). Formate dehydrogenase was from Sigma. TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) and DMPO (5,5-dimethyl-1-pyrroline-N-oxide) were purchased from Sigma-Aldrich.

Protein purification. Purification to homogeneity of the recombinant wild-type, E47A, E114A and I118S SOR proteins from *Desulfoarculus baarsii* was carried out as reported previously.[1, 2] In the purified proteins, the SOR iron active site [FeN_4S_1] was isolated in an oxygen-stable ferrous state. The SOR from *Desulfoarculus baarsii* contains an additional rubredoxin-distorded iron center [FeS_4], located at 22 Å from the iron active site.[3] In the purified protein, this iron center was in a ferric state. This rubredoxin-distorded iron center was not involved in the formation of the ferric hydroperoxo or iron-oxo species when the SORs were reacted with H_2O_2.[3-7] This [FeS_4] center was proposed to act as an electronic relay between the cellular reductases and the active site of SOR, in order to allow catalysis of superoxide reduction.[8]

Gas chromatography (GC) analysis. In a standard reaction, 0.5 to 10 mM of H_2O_2 was mixed with 1 mM of SOR, 15 mM of substrate, 4 M CH_3CN and 10 mM Tris/HCl pH 8.5, final concentrations. The reaction volume was 50 µl. The reaction was performed under a 100 % nitrogen atmosphere (inert gloves box system Jacomex), at 20 °C. Each reaction was run at least in triplicate. After 2 or 10 min incubation time, 10 µL of 10 mM benzophenone 99% diluted with dichloroethane was added as a reference. The reaction mixture was then extracted with 100 µl dichloromethane and injected into the GC apparatus. The products were identified by comparison of their GC retention times with those of authentic synthetic compounds. The gas chromatography apparatus was a PerkinElmer Autosystem (Optima-17 column, 30 m), connected to a PE Nelson 1022 integrator with flame-ionization detector. The temperature program started at 65 °C for 3 min and then increased 16 °C per min for 10 min and hold at 200 °C for 10 min.

Formate analysis. Formate was quantified from the specific reduction of NAD^+ in the presence of formate dehydrogenase (FDH, formate:NAD^+ oxidoreductase). At the end of the deformylation reaction, the sample was mixed with 750 µL of a solution containing 150 mM phosphate buffer pH 7.5, 0.2 mM pyrazole, 15 mM NAD^+ and then transferred into an UV-visible cuvette. 25 µL of a

formate dehydrogenase solution (180 mg/mL) was then added to the mixture and the increase of the absorbance at 340 nm was followed during 30-60 min until a stable value was obtained. Concentration of formate was calculated from the epsilon value of 6.3 mM^{-1} cm^{-1} for NADH at 340 nm.

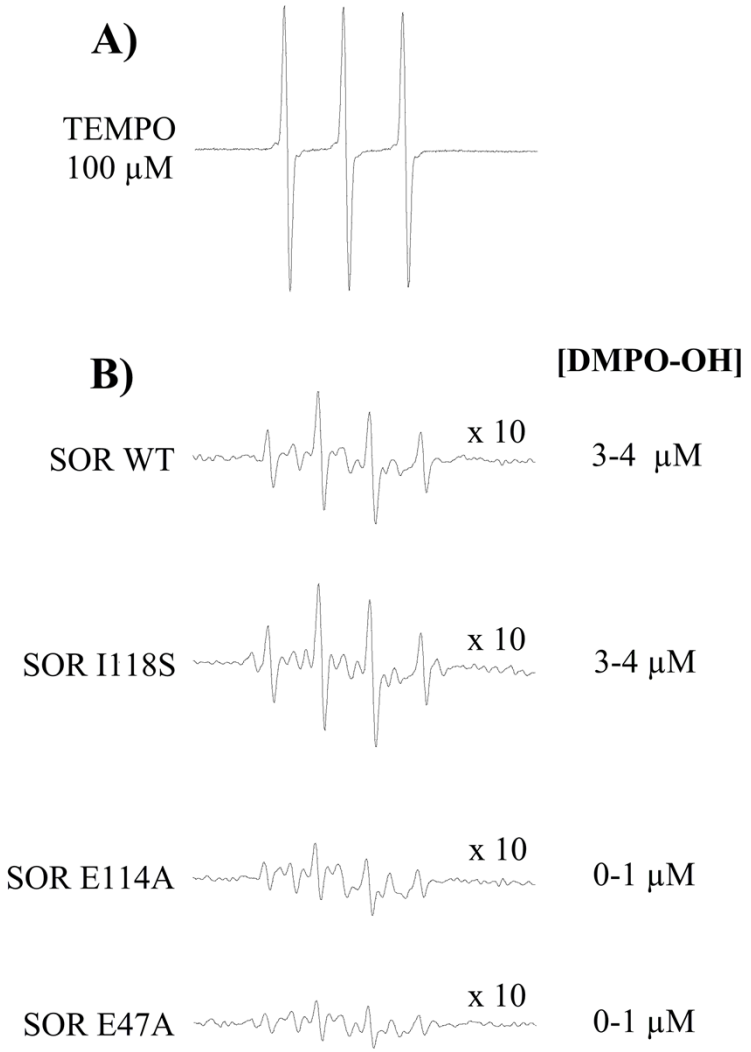
Spin trapping experiments. X-band EPR spectra were recorded on a Bruker EMX spectrometer. The microwave frequency was calibrated with a frequency counter and the magnetic field with an NMR gaussmeter. The reaction mixture was prepared in an Eppendorf tube and then transferred to an EPR-calibrated capillary tube. EPR spectra were recorded at room temperature using 2 Gauss modulation amplitude and a microwave power of 0.6 mW. The concentration of the DMPO-OH adduct was determined by integration of the EPR signal using the WIN-EPR software from Bruker and TEMPO as a reference.

2) 2-phenylpropionaldehyde deformylation

ESI Table 1. Oxidative decarboxylation of 2-phenylpropionaldehyde into acetophenone catalyzed by SOR wild-type or E47A, E114A, I118S, mutant forms (1 mM of SOR, 15 mM of 2-phenylpropionaldehyde, 10 mM Tris/HCl pH 8.5, 4 M CH_3CN) in the presence of 10 equiv H_2O_2, under N_2 atmosphere at 20 °C. The iron active site of SORs was in a ferrous state when added to the reaction mixture. After 2 min reaction time, acetophenone and benzyl formate formation were analyzed by GC.

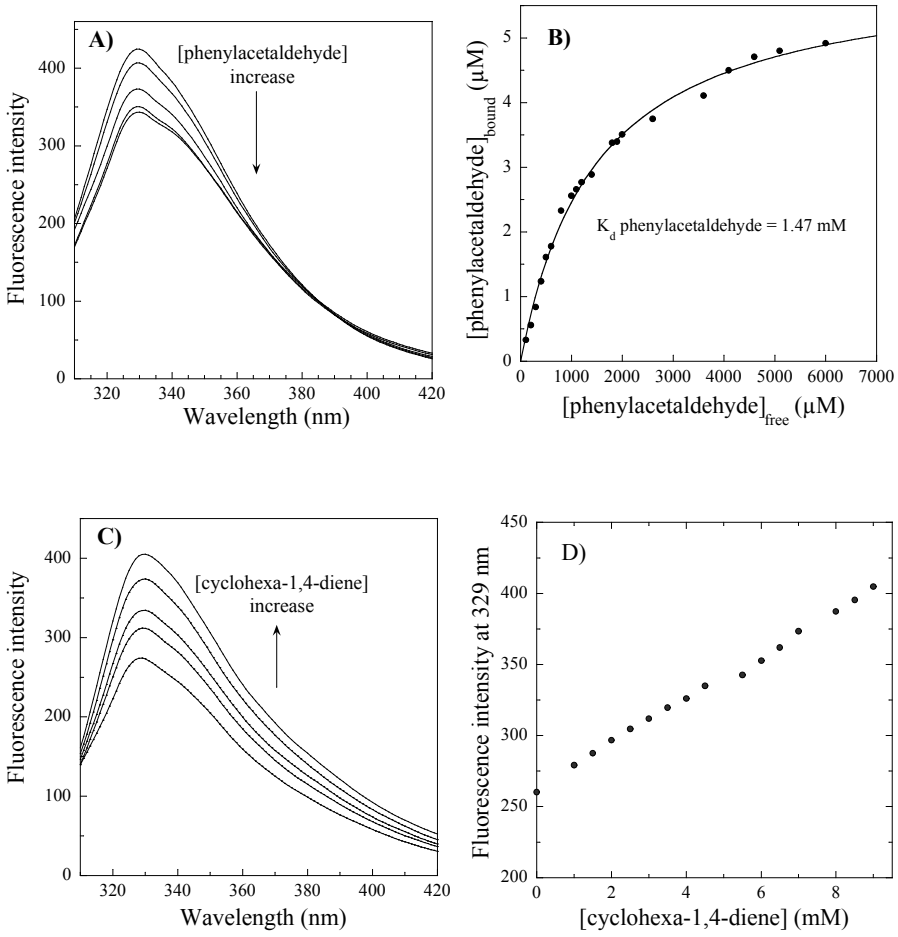
SOR forms	acetophenone (mM)	benzyl formate (mM)
wt	0.9±0.1	0.20±0.02
E47A	1.3±0.2	0.30±0.03
E114A	1.2±0.1	0.10±0.01
I118S	1.0±0.2	0.10±0.02

3) Spin trapping experiments, formation of HO•



ESI Fig. 1. Room temperature EPR spectra resulting from the reaction of the *D. baarsii* SOR proteins (active sites in a ferrous form) with H_2O_2 in the presence of the spin trap DMPO. (A) TEMPO 100 µM, used as a reference. (B) Wild-type, I118S, E114A or E47A SOR proteins (100 µM) reacted with 10 molar equivalents of H_2O_2 in the presence of 100 mM DMPO and 2 mM Tris/HCl buffer pH 8.5. Spectral intensity scalings are relative to trace (A). In all cases, room temperature EPR spectra recorded at 2 minutes after the addition of H_2O_2 showed formation of weak signals of DMPO-OH, with characteristic hyperfine coupling constants of $\alpha^N = \alpha\beta^H = 14.5$ G.[9] For all the SOR proteins, the DMPO-OH signal remained stable for at least 10 min and did not increase with H_2O_2 incubation time. In all the cases, the concentration of DMPO-OH was less than 3-4 µM.
These data show that during the reaction of the wild-type, I118S, E114A or E47A SOR proteins with H_2O_2, almost no formation of HO• radical was observed.

4) Fluorimetric titrations of SORs with various substrates



ESI Fig. 2. Fluorimetric titration of the wild-type SOR from *D. baarsii* with phenylacetadehyde and cyclohexa-1,4-diene. The fluorescence emission spectra (excitation at 295 nm) of SOR (5 µM in 10 mM Tris-HCl pH 8.5, 4 M CH_3CN,) were recorded at 20 °C using a 1-cm square cuvette, in the presence of increasing concentration of phenylacetadehyde or cyclohexa-1,4-diene. A) From the top to the bottom, after addition of 0, 1, 2.5, 4 and 5 mM of phenylacetadehyde. B) Plot of bound phenylacetadehyde as a function of free phenylacetadehyde, determined from the fluorescence variation at 329 nm. A K_d value of 1.47±0.08 mM for phenylacetadehyde was determined. C) From the bottom to the top, after addition of 0, 3, 5, 7 and 9 mM of cyclohexa-1,4-diene. D) Plot of the fluorescence variation at 329 nm as a function of [cyclohexa-1,4-diene].

ESI Table 2. K_d values for various substrates, determined from fluorimetric titrations of the different SOR forms, using the same experimental conditions than those used for the wild-type SOR with phenylacetaldehyde or cyclohexa-1,4-diene (ESI Fig. 2).

SOR forms	K_d (mM)		
	phenyl acetaldehyde	2-bromo thioanisole	cyclohex-2-en-1-ol / cyclohexa-1,4-diene
wt	1.47±0.08	2.80±0.29[a]	no K_d [b]
E47A	0.86±0.08[a]	2.70±0.24[a]	no K_d [b]
E114A	1.40±0.15[a]	2.80±0.28[a]	no K_d [b]
I118S	0.90±0.04[a]	1.95±0.17[a]	no K_d [b]

[a] the fluorescence variations of SOR in the presence of increasing concentration of the substrates was qualitatively similar to that reported in ESI Fig. 2 A,B.
[b] the fluorescence variations of SOR in the presence of increasing concentration of the substrates was qualitatively similar to that reported in ESI Fig. 2 C,D. No evidence was found for a specific interaction of the substrates with SOR and thus no K_d values could be determined.

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