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

S. Rat\textsuperscript{a,b,c}, S. Ménaire\textsuperscript{a,b,c}, F. Thomas\textsuperscript{d} and V. Nivière\textsuperscript{a,b,c,*}

Abstract: The non-heme high-spin ferric iron hydroperoxo species formed in superoxide reductase catalyzes oxidative aldehyde deformylation through its nucleophile character. This species also acts as an electrophile to catalyze oxygen atom transfer in sulfoxidation reactions, highlighting the oxidation potential of non-heme iron hydroperoxo species.

The mechanisms of oxygen activation and oxidation reactions catalyzed by metalloenzymes have been thoroughly investigated during the last past decades.\textsuperscript{1–4} For cytochrome P450\textsuperscript{5–8} and several non-heme iron monooxygenases,\textsuperscript{4, 7, 8} it is now well admitted that high-valent iron-oxo species formed at their active site is the effective oxidant for organic substrate oxidation and oxygen transfer. Nevertheless, the fact that alternative species, e.g. ferric iron (hydro)peroxo intermediate,\textsuperscript{1} or other metal-oxidant adducts,\textsuperscript{9, 10} may be also directly involved in oxidation catalysis are currently the focus of a great interest. However, although the reactivity of ferric iron hydroperoxo species has been studied on synthetic hemes\textsuperscript{11} and non-hemeiron complexes,\textsuperscript{1, 12–17} data on biological systems remain dramatically scarce, if not available. In cytochrome P450, which is one of the most studiedoxigenases, a ferric iron hydroperoxo species (compound 0) has been postulated to be the precusor of the high-valent iron species radical porphyrin (compound I).\textsuperscript{1} In spite of many efforts, compound 0 has never been directly observed experimentally, being essentially described by computational methods,\textsuperscript{18} or indirectly predicted from the analysis of reaction products.\textsuperscript{5, 19} Consequently, the reactivity of compound 0 towards substrates cannot be directly investigated in cytochrome P450. Superoxide reductase (SOR), a small non-heme mononuclear iron protein, involved in superoxide radical detoxification in some microorganisms,\textsuperscript{20–22} presents some interesting similarities with the cytochrome P450 oxygenase.\textsuperscript{23} First, both enzymes have a mononuclear iron site with a same [N\textsubscript{6}S\textsubscript{1}] coordination geometry, the sulfur axial ligand being provided by a cysteine residue in the two systems.\textsuperscript{5, 22, 24} The four nitrogen ligands come from porphyrin ring in P450,\textsuperscript{5} whereas they are provided by four histidine residues in SOR.\textsuperscript{22, 24} Second, SOR, as P450,\textsuperscript{15} also involves an iron ferric hydroperoxoide as a key intermediate in its catalytic mechanism. In SOR, this species has been trapped and thoroughly characterized. High resolution structures,\textsuperscript{20} resonance Raman (RR)\textsuperscript{25–27} and Mössbauer spectroscopies\textsuperscript{28} revealed that the hydroperoxideformed in SOR coordinates in aend-on fashion a high spin (S=5/2) iron center. Such investigations were possible because two mutations on well conserved second coordination sphere residues of the SOR from Desulfoarculusbaarsii, E47A\textsuperscript{29} and E114A\textsuperscript{30, 31} allow for an accumulation of the iron ferric hydroperoxide intermediate when the enzyme was rapidly reacted with a slight excess of H\textsubscript{2}O\textsubscript{2}. For the E47A mutant, Mössbauer studies demonstrated that in these conditions, the SOR active site was at 95% in the iron ferric hydroperoxide form.\textsuperscript{28} The E114A mutant shows RR bands associated to the iron ferric hydroperoxide with a same intensity than those characterized for theE47A mutant.\textsuperscript{31} This suggests that in the E114A mutant, the active site is also fully underan iron ferric hydroperoxide form. Interestingly, a third SOR mutant, wherein another second coordination sphere residue, I118, wasmutated into serine,\textsuperscript{29} exhibits a different reactivity. When it is incubated with H\textsubscript{2}O\textsubscript{2}, a high-valent iron-oxo species is quickly formed.\textsuperscript{32} This species presumably results from the cleavage of the O-O bond of the iron ferric hydroperoxide intermediate, which was favored in this mutant. Thus, thecontrol on the nature of the oxygen adducts (iron ferric hydroperoxide or iron-oxo species)in theSOR mutants opens the door for comparative reactivity studies on oxidation reactions.

In order to investigate the reactivity of the SOR ferric hydroperoxide intermediate, we tested the ability of the D. baarsiiSOR wild-type, E47A, E114A and I118S mutant forms to perform oxidative aldehyde deformylationin the presence of H\textsubscript{2}O\textsubscript{2}(ESI). This reaction that requires a nucleophile attack on the aldehyde was reported to be carried out by ferric iron (hydro)peroxo species.\textsuperscript{12, 13, 31} Whereas the electrophilic iron-oxo species would oxidize aldehydes into their corresponding acid.\textsuperscript{13} The reaction was conducted under anaerobic conditions, in the presence of CH\textsubscript{3}CN (4 M) in order to ensure a sufficient solubility of the organic substrates (Fig. 1). We observed that CH\textsubscript{3}CN at the concentration of
4 M did not affect the stability of SOR, at least during 30 min at room temperature (data not shown). In the absence of SOR, no oxidation products were detected by GC. In the presence of SOR, phenylacetaldehyde was oxidized into benzaldehyde as a majority, without formation of phenylacetic acid (Fig. 1). The yield of benzaldehyde was about 20-37 % with respect to the SOR forms, in the presence of 1 molar equivalent of H₂O₂ (Fig. 1).

The fact that benzaldehyde, formate and benzyl formate (Table 1) could be detected as products suggests that the reaction mechanism is similar to that proposed for the deformylation reaction catalyzed by the ferric (hydro)peroxy intermediate in cytochrome P450 (Scheme 1).13 In this mechanism, anucleophilic addition of the ferric (hydro)peroxy species on the aldehyde affords a peroxyhemiacetal adduct, which then undergoes a radical fragmentation to generate formate. The transient Fe³⁺-O²⁻ species, and also possibly H₂O₂ still present in the solution, could further oxidize the phenyl methyl radical intermediate into benzaldehyde, without accumulation of benzyl alcohol. That such a phenyl methyl radical species was transiently generated during the reaction of SOR with phenyl-acetaldehyde was confirmed by performing the reaction in the presence of an excess of BrCCl₃. In these conditions PhCH₂Br, which results from the quenching of the phenyl methyl radical species by BrCCl₃, was detected by GC analysis, whereas production of benzaldehyde was significantly decreased (Table 1, Scheme 1). Finally, the formation of a small amount of benzyl formate might be explained as follows: the phenyl methyl radical could attack the carbonyl oxygen of the formic acid to give a carbon-centered radical that could be further hydroxylated by the Fe³⁺-O²⁻ or Fe-OOH species. It would follow a rearrangement and an elimination of a water molecule (Scheme 1). Alternatively, as proposed in reference 10, benzyl formate might result from an oxidation of the phenyl methyl radical into a carbocation species and a further reaction with formate. Note that as shown in Table 1, the mass balance is not equilibrated. Since a radical chemistry is involved, it is suspected that part of the radical intermediate reacted with the buffer and/or with the polypeptide chain.

With 10 equiv. of H₂O₂ up to 2.5 turnovers were observed in the case of the E47A SOR mutant (Fig. 1). Increasing the reaction time to 10 min did not affect the yield in benzaldehyde, showing that the reaction was completed within 2 min. In the presence of 10 equiv. of H₂O₂ (Table 1), i.e. the conditions that yielded the highest amount of benzaldehyde, formate production was observed. The relative formate amounts were 30, 26, 52 and 64 % of that of benzaldehyde, for the wild-type, I118S, E114A and E47A SOR forms, respectively (Table 1). GC analysis also evidenced formation of benzyl formate with all the SOR forms, in an amount of about 10-20 % of that of benzaldehyde (Table 1). When the reaction was carried out with 10 equiv. of H₂O₂, analysis of the products by mass spectrometry showed that 87±8 % of the oxygen incorporated onto benzaldehyde product was originated from the ¹⁸O-labeled peroxide. The small amount of ¹⁶O labeled formic acid might originate from oxygen exchange of the transient Fe³⁺-O²⁻ species (see below) with the solvent.

2-phenylpropionialdehyde, a more sterically hindered substrate, was also deformylated into acetophenonyl SOR (ESI). The yield was nevertheless much lower than that determined for phenylacetaldehyde (ESI).

Altogether, these data showed that the SOR from D. baarsii carries out oxidative decarboxylation of aldehydes in the presence of H₂O₂ (Scheme 1). The results demonstrate that the ferric iron hydroperoxide species, which is formed in high yield in the E47A,15,28 and E114A24,27 SOR mutants, acts as a catalytically competent nucleophile. Furthermore, HO⁺ radical could not be involved in this process, since no formation of HO²⁻ was observed by spin trapping during the reaction of SOR with H₂O₂ (ESI).

Interestingly, the ferric iron hydroperoxide species was formed in a lower yield with the wild-type protein,26 which is in line with its lower activity to oxidize aldehyde (Fig. 1). The I118S SOR mutant, which was recently shown to form an iron-oxo species in the presence of H₂O₂,29 was also able to carry out oxidative decarboxylation of aldehydes, albeit in a lower yield compared to the E47A and E114A SOR mutants (Fig. 1). Based on RR studies, it was proposed that the iron-oxo species formed in the I118S SOR mutant results from a rapid O-O bond cleavage of a transiently formed iron

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**Table 1.** Reaction products for the deformylation of phenylacetaldehyde in the presence of 10 equiv of H₂O₂. The experimental conditions are the same than in Fig. 1. Benzaldehyde, benzyl formate and PhCH₂Br were quantified by GC. Formate was quantified with a formate dehydrogenase assay (ESI).

<table>
<thead>
<tr>
<th>SOR forms</th>
<th>benzaldehyde (mM)</th>
<th>benzyl formate (mM)</th>
<th>PhCH₂Br (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No BrCCl₃</td>
<td>0.30±0.03</td>
<td>1.10±0.02</td>
<td>0.34±0.01</td>
</tr>
<tr>
<td>E47A</td>
<td>1.60±0.20</td>
<td>2.54±0.02</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>E114A</td>
<td>1.40±0.10</td>
<td>2.14±0.02</td>
<td>0.19±0.02</td>
</tr>
<tr>
<td>I118S</td>
<td>0.40±0.04</td>
<td>1.50±0.3</td>
<td>0.04±0.01</td>
</tr>
</tbody>
</table>

**Table 2.** Reaction products for the deformylation of phenylacetaldehyde in the presence of 10 equiv of H₂O₂. The experimental conditions are the same than in Fig. 1. Benzaldehyde, benzyl formate and PhCH₂Br were quantified by GC. Formate was quantified with a formate dehydrogenase assay (ESI).

**Figure 1.** Oxidative decarboxylation of phenylacetaldehyde into benzaldehyde catalyzed by the D. baarsii SOR wild-type, E47A, E114A or I118S, mutant forms (1 mM of SOR, 15 mM of phenylacetaldehyde, 10 mM T/HCl pH 8.5, 4 M CH₃CN) in the presence of 0.5-10 equiv. H₂O₂, under N₂ 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, benzaldehyde formation was analyzed by GC.

**Scheme 1.** Proposed mechanism for the deformylation reaction catalyzed by the ferric iron hydroperoxide species formed in SOR, as described in reference 10.
hydroperoxide. This suggests the existence of two competitive pathways that consume the iron hydroperoxide species in this SOR mutant: conversion into the iron-oxo species or nucleophilic oxidation of aldehyde.

Oxidation of thioanisole was investigated in similar conditions than those used for deformylation (Fig. 2). No oxidation products of thioanisole could be detected by GC in the absence of SOR. In the presence of SOR and 1 mol-equiv. of H₂O₂, thioanisole was oxidized into sulfoxide, in yields of 60 and 72 % with respect to SOR for the wild-type and I118S proteins, respectively. The yield was slightly lower for the E47A and E114A mutants, 42 and 36 %, respectively (Fig. 2). In the presence of 10 equiv. of H₂O₂, the number of turnovers reached 2 with the wild-type and E47A SOR forms (Fig. 2). In all the cases, the reaction was completed within 2 min and no other oxidation products, e.g. sulfone, were detected by GC.

2-bromothioanisole and 2-methylthionaphthalene were also oxidized into sulfoxide by the four different SOR forms (ESI), albeit with a much slower yield than thioanisole. For 2-bromothioanisole, this could reflect an electronic deactivation of the sulfide by the bromine, whereas for 2-methylthionaphthalene, this could be due to its larger steric hindrance compared to thioanisole.

These data show that the wild-type, E47A, E114A and I118S SORs catalyze sulfoxidation of thioanisole in a comparable extent. No evidence supporting the formation of iron-oxo species in the presence of H₂O₂ were reported for the wild-type, E47A and E114A SORs. Thus, these results strongly suggest that the ferric iron hydroperoxide species is the active species for sulfoxidation of thioanisole. For the I118S SOR mutant, the ferric iron hydroperoxide species, which was rapidly formed in the presence of H₂O₂, might also be involved in the oxidation of thioanisole into sulfoxide. Nevertheless, we cannot exclude that the iron-oxo species formed in this mutant was also involved in the sulfoxidation reaction.

Altogether, these data demonstrate that the ferric iron hydroperoxide species formed in the SOR active site can both act as a nucleophile for oxidative decarboxylation of aldehyde, and as an electrophile to catalyze sulfoxidation of sulfides.

Finally, the electrophilic character of the ferric iron hydroperoxide and iron-oxo species formed in SOR mutants was also investigated in the epoxidation of styrene, cyclohex-2-en-1-ol, cyclooctene, and for the oxidation of weak C-H bonds, with xanthene, 9,10-dihydro-anthracene, fluorene, cyclohexa-1,4-diene and ethylenbenzene. In the presence from 0.5 to 10 equiv. of H₂O₂, we were unable to detect any oxidation product by GC with the four SOR forms (data not shown). Note that these cycloalkanes were tested at a concentration of 15 mM. Attempts to increase their concentrations beyond 15 mM in the reaction mixture initiated precipitation, while acetonitrile concentration higher than 4 M led to SOR instability.

It was rather unexpected that the iron-oxo species formed in the I118S SOR mutant was not potent for oxidation of any of the tested substrates. This result could be interpreted as a lack of activity in the presence of increasing concentration of phenylacetaldehyde and 2-bromothioanisole (thioanisole is not oxidized) in the SOR, but rather due to the soluble nature of the substrate. The SOR from D. baarsii indeed contains atryptophan residue located at about 8 Å from the iron site, which could be used as a probe to monitor interactions of putative substrate with the active site. In the presence of increasing concentration of phenylacetaldehyde and 2-bromothioanisole, the fluorescence of SOR was quenched in a saturable manner (ESI). These data are consistent with a specific binding of these two substrates (which were oxidized by SOR, see above) to the active site. The calculated Kᵣ values are about 1 mM for phenylacetaldehyde and 3 mM for 2-bromothioanisole (ESI). A dramatically different behaviour was observed in the case of cyclohex-2-en-1-ol or cyclohexa-1,4-diene, which were not oxidized by SOR (ESI). Upon addition of increasing amounts of these substrates, the fluorescence increased linearly, without evidence for saturation. This change of fluorescence likely reflects a modification of the polarity of the solvent due to the addition of organic molecules rather than a specific interaction of these molecules with SOR. These data suggest that the alkylation compounds cannot interact with the polaractive site of SOR. Regarding the aromaticphenylacetaldehyde and thioanisole, which are oxidized by SOR, the aldehyde or sulfide groups would reach more easily the active site, allowing reaction with the oxidant species. These data illustrate the major impact of the substrates binding site on the enzyme reactivity.

**Conclusion**

In summary, we report for the first time the reactivity of a non-hememononuclear high-spin ferric iron hydroperoxo species in a [S₅N₄] coordination set. This coordination set provided by SOR is reminiscent of that of cytochrome P450. However, the four equatorial ligands are histidine donors in SOR instead of pyrroles from porphyrin cofactor in P450 and the electric configuration of the iron center is high-spin in the iron hydroperoxo species of SOR instead of low-spin in P450. We showed that the high-spin iron hydroperoxo species formed in SOR catalyse deformylation of aldehydes, demonstrating its ability to carry out oxidation through its nucleophilic character. In addition, it can act as an electrophilic species to catalyze oxygen atom transfer sulfoxidation reactions. Attempt to study its reactivity towards C-H bond oxidation were not yet possible, most likely due to the polar environment of the SOR iron site, which prevents interactions with the alkylationamine substrate tested here. The dual character of the SOR dioxygen adduct confirms the existence of the Fe₄⁵⁵-OOH reaction type.

Recently, a high-spin non-heme mononuclear iron hydroperoxo complex bearing a tetradeinate N₄ ligand was shown to carry out nucleophilic deformylation and electrophilic oxidations. Our present studies extend the reactivity of the iron hydroperoxo species...
to a [N₄S₁] coordination sphere in a non-heme protein environment and provide new evidence for the important role thatsuch a species could play in bio catalysis, especially in oxidation reactions.

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**Notes and references**