Hydrogen bonding to the cysteine ligand of superoxide reductase: acid–base control of the reaction intermediates

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Abstract  Superoxide reductase (SOR) is a non-heme iron metalloenzyme that detoxifies superoxide radical in microorganisms. Its active site consists of an unusual non-heme Fe²⁺ center in a [His₄Cys₁] square pyramidal pentacoordination, with the axial cysteine ligand proposed to be an essential feature in catalysis. Two NH peptide groups from isoleucine 118 and histidine 119 establish hydrogen bonds involving the sulfur ligand (Desulfoarculus baarsii SOR numbering). To investigate the catalytic role of these hydrogen bonds, the isoleucine 118 residue of the SOR from Desulfoarculus baarsii was mutated into alanine, aspartate, or serine residues. Resonance Raman spectroscopy showed that the mutations specifically induced an increase of the strength of the Fe³⁺–S(Cys) and S–Cβ(Cys) bonds as well as a change in conformation of the cysteinyl side chain, which was associated with the alteration of the NH hydrogen bonding involving the sulfur ligand. The effects of the isoleucine mutations on the reactivity of SOR with O₂⁻ were investigated by pulse radiolysis. These studies showed that the mutations induced a specific increase of the pKₐ of the first reaction intermediate, recently proposed to be an Fe²⁺–O₂⁻ species. These data were supported by density functional theory calculations conducted on three models of the Fe²⁺–O₂⁻ intermediate, with one, two, or no hydrogen bonds involving the sulfur ligand. Our results demonstrated that the hydrogen bonds between the NH (peptide) and the cysteine ligand tightly control the rate of protonation of the Fe²⁺–O₂⁻ reaction intermediate to form an Fe³⁺–OOH species.

Keywords  Superoxide reductase · Hydrogen bonds · Sulfur ligand · Mononuclear iron site · Catalytic mechanism

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Introduction

Superoxide radical (O$_2^{•−}$), the one-electron reduction product of oxygen, is a side product of aerobic metabolism [1, 2]. It is considered to be the first reactive oxygen species formed within the cells, initiating toxic oxidative stress processes which dramatically affect cellular metabolism and viability [1, 2]. It is now well documented that high levels of oxidative stress in human cells are involved in the development of serious diseases, such as cancers and diseases related to aging, such as Alzheimer disease and Parkinson disease [3]. Detoxification of O$_2^{•−}$ is thus a crucial part of the cellular antioxidant defense mechanisms which allow the cells to cope with the presence of molecular oxygen [1, 2]. So far, only two superoxide detoxification systems have been described. The first one is the well-known superoxide dismutase, present in almost all aerobic organisms, catalyzing dismutation of O$_2^{•−}$ into H$_2$O$_2$ and O$_2$ [4]. The second one, superoxide reductase (SOR), was discovered more recently [5, 6] and up to now has been only found in microorganisms [7–10]. Superoxide dismutase and SOR are structurally unrelated metalloproteins and carry out different reactions. SOR catalyzes the one-electron reduction of O$_2^{•−}$ to H$_2$O$_2$ (Eq. 1), the electron being provided by cellular reductases or soluble electron transfer proteins [7–10]:

$$\text{O}_2^{•−} + 1e^{-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2.$$  (1)

Although different classes of SOR have been described depending on the presence of an additional structural domain, which can bind a rubredoxin-like iron center (named center I), they all exhibit the same active site, named center II [7–10]. The SOR active site in its reduced form consists of an unusual non-heme Fe$^{2+}$ center in a [His$_4$Cys$_1$] square pyramidal pentacoordination [11–14], which reacts at a nearly diffusion controlled rate with O$_2^{•−}$ [15–19]. The catalytic mechanism of SORs has been extensively studied, mainly using pulse radiolysis [15–19]. Ferric iron hydroperoxide [14–18] and more recently ferrous iron–superoxo species [19, 20] have been proposed as reaction intermediates during the course of superoxide reduction to produce H$_2$O$_2$. A first reaction intermediate (T1) is rapidly formed on the binding of O$_2^{•−}$ to the vacant sixth coordination position of the Fe$^{2+}$ site (Scheme 1). In the case of the Desulfoarculus baarsii enzyme, T1 was proposed to be a ferrous iron–superoxo species [19]. This first reaction intermediate undergoes protonation by H$_3$O$^+$ from the bulk solvent, to form a second reaction intermediate, an Fe$^{3+}$–hydroperoxo species (T1’ in Scheme 1). The second reaction intermediate was proposed to be specifically protonated on its proximal oxygen atom by a water molecule, hydrogen-bonded to K48, a highly conserved residue located close to the iron site, thus promoting the formation of the H$_2$O$_2$ product [14, 19] (Scheme 1). Although this second reaction intermediate could not be observed by pulse radiolysis, probably because it did not accumulate [19], its presence was deduced both from the X-ray structures of iron–peroxide intermediates trapped in the active site of SOR [14] and from the effects of the K48I mutation on the reactivity of SOR [19]. In particular, it was proposed that the mutation of K48 into isoleucine, by preventing a specific protonation of the proximal oxygen of the Fe$^{3+}$–OOH species, favors cleavage of its O–O bond to form a high-valent iron–oxo species, which is avoided in the wild-type enzyme [19]. Finally, following the release of H$_2$O$_2$, the Fe$^{3+}$ active site remains hexacoordinated, with the HO$^-$/H$_2$O ligand in the sixth coordination position (P1 in Scheme 1). The HO$^-$/H$_2$O ligand is further replaced by the carboxylate side chain of the conserved E47 residue, to form a hexacoordinated Fe$^{3+}$–E47 species at the end of the catalytic cycle [17, 19] (P2 in Scheme 1).

The strictly conserved cysteine axial ligand in the SOR active site has been hypothesized to be an essential feature for the reactivity of the enzyme with superoxide [7–10]. However, cysteine substitution to probe its function is not possible, since this would lead, most likely, to the formation of a demetalated SOR active site [21]. Interestingly, several amino acid residues were pointed out to interact with the sulfur atom of the cysteine ligand, suggesting that they could modulate the properties of the S–Fe bond and thus the reactivity of SOR with O$_2^{•−}$. In D. baarsii SOR, the buried carboxylic side chain of E114 establishes a dipolar interaction with the sulfur ligand (C116) [22, 23], and its mutation into an alanine (E114A) was shown to specifically decrease the strength the Fe–S(Cys) bond [23]. Such a modification of the strength of the Fe–S(Cys) bond was reported to induce a decrease of the pK$\_a$ of the first reaction intermediate T1, providing experimental evidence for a role of the sulfur ligand in the control of the evolution of the reaction intermediate in SOR catalysis [23].

By comparison with other Fe–S(Cys)-containing metalloproteins, cytochrome P450, rubredoxins, or ferredoxins [24–26], it was proposed that in SORs, two NH peptide groups from leucine/isoleucine and histidine of a well-conserved C116–N117–L/I118–H119 tetrapeptide are within hydrogen-bonding distance from the sulfur iron.

Abbreviations

| Abbreviation | Full Form |
|--------------|-----------|
| CT | Charge transfer |
| DFT | Density functional theory |
| FTIR | Fourier transform IR |
| PDB | Protein Data Bank |
| SOR | Superoxide reductase |
| Tris | Tris(hydroxymethyl)aminomethane |

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ligand \[11–14, 27\] (Fig. 1). In the crystal structure of the SOR from \textit{Desulfoarculus baarsii}, these N(H)–S distances are 3.4 and 3.2 Å for the I118 and H119 backbone, respectively (Fig. 1), in agreement with the fact that hydrogen bonds involving a sulfur atom are longer than those involving an oxygen atom. These N(H)–S bond distances in SOR are slightly shorter than those reported for the sulfur ligands of rubredoxin (3.6–3.9 Å)[26].

In this work, we have investigated the effect of one of the peptide NH groups on the sulfur ligand and on the reactivity of the SOR from \textit{D. baarsii} with O$_2$•$^-$ by mutating the I118 residue into alanine, aspartate, or serine. Our data show that mutations of I118 induce a strengthening of the Fe–S(Cys) and S–C$_b$(Cys) bonds and an increase of the pK$_a$ of the reaction intermediates. These results are consistent with the decrease of the strength of a hydrogen bond between the I118 peptide NH group and the sulfur ligand. These effects are opposite to those reported for the E114A mutant [23], showing that, in the SOR from \textit{D. baarsii}, the strength of the Fe–S(Cys) bond is finely tuned by different second coordination sphere residues.

**Materials and methods**

**Materials**

For pulse radiolysis experiments, sodium formate and buffers were of the highest grade available (Prolabo Normatom or Merck Suprapur). Oxygen was from ALPHA GAZ. Its purity was higher than 99.99 %. Water was purified using an Elga Maxima system (resistivity 18.2 MΩ). K$_2$IrCl$_6$ was from Strem Chemicals.

**Site-directed mutagenesis and protein purification**

Mutagenesis was conducted on the plasmid pMJ25 [6], with a QuikChange® site-directed mutagenesis kit from Stratagene. Plasmid pMJ25 is a pJF119EH derivative, in which the \textit{sor} gene from \textit{D. baarsii} was cloned under the control of a \textit{tac} isopropyl $\beta$-D-thiogalactopyranoside inducible promotor [6]. Six primers were designed for the PCR-based site-directed mutagenesis to create the three intermediates (T1'), a ferric iron–hydroperoxo species, was not observed experimentally, but was deduced from studies on the reactivity of the K48I SOR mutant with O$_2$•$^-$ [19] and from the crystal structures of iron–hydroperoxide species trapped in the active site of SOR [14].
I118 SOR mutants, I118A, I118S, and I118D. Primer 1 (5'-GAA TAC TGC AAC GCC CAC GCC CAC TGG 3') and primer 2 (5’CCA GTG GCC GTG GCC GTT GCA GTA TTC 3') were used for the I118A mutation. Primer 3 (5'-GAA TAC TGC AAC GAC CAC GCC CAC TGG 3') and primer 4 (5’ CCA GTG GCC GTG GTC GTT GTA GTA TTC 3') were used for the I118D mutation. Primer 5 (5'-GAA TAC TGC AAC AGC CAC GCC CAC TGG 3') and primer 6 (5’ CCA GTG GCC GTG GTC GTT GTA GTA TTC 3') were used for the I118S mutation. The mutations were verified by DNA sequencing. The resulting plasmids, pTE118A, pTE118S, and pTE118D, were transformed in Escherichia coli BL21. Overexpression and purification of the I118 mutant proteins were performed as reported for the wild-type protein [6].

The purified mutant proteins were as stable as the wild-type protein. The UV–visible absorption spectrum of the purified proteins exhibited an A280 nm/A503 nm ratio of 4.8–4.9 and appeared to be homogeneous, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis (data not shown). Protein concentrations were determined using the Bio-Rad protein assay reagent. For each SOR mutant, full metalation of the two mononuclear iron sites was verified by atomic absorption spectroscopy, with two iron atoms per polypeptide chain (data not shown). All the I118 mutant proteins were isolated with an oxidized center I ([Fe4S4]2+) and a fully reduced center II. Fully oxidized SORs were obtained by treatment of the proteins with a slight molar excess of K2IrCl6.

pH studies

The following buffers were used to cover a pH range from 5.0 to 10.5: acetate for pH 5.0 and 5.5; 2-morpholinoethanesulfonic acid for pH 6.0, 6.5; 1,3-bis(tris(hydroxymethyl)methylamino)propane or N(2-hydroxyethyl)piperazine-N’-ethanesulfonic acid for pH 7.0; tris(hydroxymethyl)aminomethane (Tris)–HCl for pH 7.6, 8.1, 8.5, and 8.8; and glycine–NaOH for pH 9.1, 9.5, 10.2, and 10.5. The apparent pH a of the alkaline transition was determined from the pH dependence of the absorbance at 660 nm of the fully oxidized SOR mutant proteins, as reported in [16]. The titration curve was fitted with the equation expected from a single protonation process: 

\[ A_{660 \text{ nm}} = (A_{660 \text{ max}} + A_{660 \text{ min}} \times 10^{(pH - pK_a \text{ app})}) / (1 + 10^{(pH - pK_a \text{ app})}). \]

Redox titrations

The redox titrations were monitored by UV–visible absorption spectroscopy at 291 K under an \( \text{N}_2 \) atmosphere in a Jacoemx glove box equipped with a UV–visible cell coupled to a Uvikon XL spectrophotometer by optical fibers (Photometrics system). The sample cuvette contained 3 mL of 50 \( \mu \)M protein solution in 50 mM Tris–HCl buffer, pH 7.6, in the presence of the following mediators each at 2 \( \mu \)M: ferrocene (+422 mV), \( \text{N}^{+} \cdot \text{dimethyl-p-phenyl-enediamine} \) (+371 mV), 1,4-benzoquinone (+280 mV), 2,5-dimethyl-p-benzoquinone (+180 mV), and duroquinone (+5 mV). The redox potentials were measured with a combined Pt–Ag/AgCl/KCl (3 M) microelectrode, with respect to the standard hydrogen electrode. The potential of the solution was poised by stepwise additions of small quantities of either 5 mM \( \text{K}_2\text{IrCl}_6 \) (starting from as-isolated SOR) or 10 mM sodium dithionite (starting from oxidized SOR). After each addition of \( \text{K}_2\text{IrCl}_6 \) or sodium dithionite, the solution was equilibrated for 5 min. After the potential reading had stabilized, the potential and the UV–visible absorption spectrum were recorded. The data were fitted to the Nernst equation for a one-electron reaction.

Pulse radiolysis

Pulse radiolysis measurements were performed as described elsewhere [28]. Briefly, free radicals were generated by irradiation of \( \text{O}_2 \)-saturated aqueous protein solutions (100 \( \mu \)M) in 2 mM buffer, 10 mM sodium formate with 0.2–2-\( \mu \)s pulses of 4-MeV electrons at the linear accelerator at the Curie Institute (Orsay, France). \( \text{O}_2^* \) was generated during the scavenging by formate of the radiolytically produced hydroxyl radical, \( \text{HO}^* \). [28]. The doses per pulse were calibrated from the absorption of the thiocyanate radical (SCN)2•– obtained by radiolysis of the thiocyanate ion in \( \text{N}_2\text{O}_5^- \) saturated solution (10–2 M SCN–). \( G[(\text{SCN})_2^*] = 0.55 \mu \text{mol J}^{-1}, \epsilon_{472 \text{ nm}} = 7,580 \text{ M}^{-1} \text{ cm}^{-1} \) for instance, a dose of 5 Gy per pulse (0.2 \( \mu \)s long) resulted in 2.8 \( \mu \)M O2•–. The dose varied linearly with the pulse length. Reactions were followed spectrophotometrically, using a Hamamatsu SuperQuiet xenon–mercury lamp (150 W) between 310 and 750 nm or a tungsten lamp between 450 and 750 nm, at 293 K in a 2 cm path length fused-silica cuvette. In all the pulse radiolysis experiments, a cutoff filter cutting off all wavelengths below 425 nm was positioned between the lamp and the cuvette. Kinetics were analyzed at different wavelengths using a Levenberg–Marquardt algorithm from the KaleidaGraph® software package (Synergy Software).

Resonance Raman spectroscopy

 For the Raman experiments, 3.5 \( \mu \)L of concentrated protein (4–6 mM) was deposited on a glass slide sample holder and then transferred into a cold helium gas circulating optical cryostat (STVP-100, Janis Research), held at 15 K. Resonance Raman spectra were recorded using a Jobin–Yvon
U1000 spectrometer equipped with a liquid-nitrogen-cooled CCD detector (Spectrum One, Jobin–Yvon, France). Excitation at 647.1 nm (30 mW) was provided by an Innova Kr+ laser (Coherent, Palo Alto, CA, USA). The signal-to-noise ratios were improved by spectral collections of six cycles of 30-s accumulation time. The reported spectra were the results of averaging five to seven single spectra. The spectral analysis was done using GRAMS/32 (Galactic Industries). The spectrometer calibration was done as described in [29]. The frequencies of the resonance Raman bands of SORs were also internally calibrated against the main band of the ice lattice (230 cm\(^{-1}\)) and a residual laser emission at 676.4 nm (669 cm\(^{-1}\)). The frequency precision was 0.5–1 cm\(^{-1}\) for the most intense bands and 1.5–2 cm\(^{-1}\) for the weakest bands.

Fourier transform IR spectroscopy

Electrochemically induced Fourier transform IR (FTIR) difference spectra were recorded, using a short path length electrochemical cell and a Bruker 66 SX spectrometer equipped with a KBr beam splitter and a nitrogen-cooled cooled CCD detector (Spectrum One, Jobin–Yvon, France). Excitation at 647.1 nm (30 mW) was provided by an Innova Kr+ laser (Coherent, Palo Alto, CA, USA). The signal-to-noise ratios were improved by spectral collections of six cycles of 30-s accumulation time. The reported spectra were the results of averaging five to seven single spectra. The spectral analysis was done using GRAMS/32 (Galactic Industries). The spectrometer calibration was done as described in [29]. The frequencies of the resonance Raman bands of SORs were also internally calibrated against the main band of the ice lattice (230 cm\(^{-1}\)) and a residual laser emission at 676.4 nm (669 cm\(^{-1}\)). The frequency precision was 0.5–1 cm\(^{-1}\) for the most intense bands and 1.5–2 cm\(^{-1}\) for the weakest bands.

Electronic absorption and EPR spectroscopies

Optical absorbance measurements were performed using a Varian Cary spectrophotometer (0.2-nm bandwidth) with a 1 cm path length cuvette. Low-temperature (4.2 K) X-band EPR spectra were recorded with a Bruker EMX 081 spectrometer equipped with an Oxford Instruments continuous-flow cold helium gas cryostat.

Density functional theory calculations

Three models (designed A, B, and C) of the SOR active site were built using the structure of the wild-type SOR with the Protein Data Bank (PDB) code 2JI1 [14]. All include the side chains plus the C\(\alpha\) atoms of the five amino acids binding the iron atom (residues H49, H69, H75, H119, and C116). In model A, \(\alpha\)-carbons were kept fixed in their crystallographic positions, whereas the hydrogens bound to them (replacing the adjacent atoms) were free to move only along the C\(\alpha\)-N or C\(\alpha\)-C direction found in the crystallographic structure. Model B is similar to model A except that a peptide bond between H119 and I118 was added (i.e., an –NH–COOH moiety replaces the hydrogen atom of model A). To maintain the orientation of the N – H⋅⋅⋅S hydrogen bond, the additional carbonyl oxygen was fixed in its crystallographic position, as reported in [30]. In model C, the backbone of the C116–N117–I118–H119 tetrapeptide was added. The same C\(\alpha\) atoms as in models A and B were kept fixed, as well as the two carbonyl oxygens in order to maintain the two N – H⋅⋅⋅S interactions. For residues N117 and I118, side chains were replaced by hydrogen atoms in order to save computation time. Each model was optimized in the absence or presence of superoxide anion or hydroperoxide bound to the iron atom. A model was also considered with a hydroxide anion bound to iron, as a model of the Fe\(^{3+}\) resting state. Calculations were performed using the Gaussian 03 package [31], and geometries were optimized with the B3LYP [32, 33] hybrid density functional using a double-\(\zeta\) quality basis set (hereafter B1). The 6-31G* basis set was used for all atoms but iron and nitrogen, oxygen, and sulfur of the first coordination sphere of the iron. For these nitrogen, oxygen, and sulfur atoms of the first coordination sphere of the iron and involved in hydrogen bonds, the 6-31+G* basis set was used. For iron, lan12dz with an effective core potential [34] was completed by a set of \(f\) polarization functions of exponent 2.462 and a set of \(d\) diffuse functions of exponent 0.0706 [35]. Energies were computed with B3LYP and the all-electron triple-\(\zeta\) 6-311+G** (called B2 in the following) basis set on previously optimized geometries. Polarization effects on the environment were accounted for by the means of the implicit polarizable continuum model of the solvent [36], with a dielectric constant of 5.75 and a probe radius of 2.7, at the B1 level. The proton affinity of the different models was explored by computing the energetics of the reaction leading to the protonated counterpart. This was done by using a solvation energy of 260 kcal mol\(^{-1}\) [37]. All systems were studied in their high-spin state (\(S = 2\) for the Fe\(^{2+}\) resting state, \(S = 5/2\) for Fe\(^{2+}\)–O\(_2\)\(^{–}\), Fe\(^{3+}\)–OH, and Fe\(^{3+}\)–OH), consistent with previous results obtained on similar models using comparable methods [19, 20, 30].

Results

Electronic absorption spectroscopy

The UV–visible absorption spectra of the purified \(D.\) baarsii I118A, I118D, and I118S SOR mutants exhibited characteristic absorption bands at 370 nm and 503 nm (\(\epsilon_{503\text{ nm}} = 4.4\) mM\(^{-1}\) cm\(^{-1}\)) (data not shown), arising from ferric iron center I [6]. When the mutants were treated with K\(_2\)IrCl\(_6\), the spectra exhibited an increase in absorbance in the 500–700-nm region, reflecting oxidation of
iron center II [16]. As reported for the wild-type SOR [16], the absorption band maximum of ferric iron center II of the I118 mutants was blueshifted at alkaline pH (Fig. S1). This process, called alkaline transition, reflects the displacement of the E47 ligand at the sixth coordination position of center II (Fe3+–E47, maximum absorbance at 644 nm) by a hydroxide ion, to form an Fe3+–OH species (maximum absorbance at 560 nm) [16, 39]. As shown in Table 1, when compared with the wild-type SOR, the apparent pK𝑎 associated with this process was significantly higher in the I118 SOR mutant. This effect was less pronounced for the I118A and I118D mutants. In addition, the absorption band maxima of the Fe3+–E47 forms of the I118A, I118D, and I118S SOR mutants were shifted by +7 nm (−167 cm−1), +4 nm (−96 cm−1), and +7 nm (−167 cm−1), respectively, when compared with the wild-type SOR (Table 1). Since the SOR proteins were found to be highly unstable at pH values higher than 10.2, the absorption band maxima of the Fe3+–OH forms of the I118 mutants could not be determined, but from the spectra recorded at pH 10.2 we deduced that they were below 580 nm (Fig. S1).

### EPR spectroscopy

The 4 K EPR spectra of the as-isolated I118 SOR mutants displayed resonances at g = 7.7, 5.7, 4.1, and 1.8, similar to those obtained for the wild-type protein [6] (data not shown). These resonances were typical of those of an Fe3+ ion in a distorted tetrahedral FeS₄ center and originated from center I [6, 27]. The 4K EPR spectrum of the I118 SOR mutants oxidized with a slight excess of K₂IrCl₆ exhibited an additional signal at g = 4.3, identical to that observed for the oxidized wild-type SOR (data not shown). This g = 4.3 signal was attributed to the oxidized center II, with a rhombic (E/J = 0.33) high-spin (S = 5/2) ferric iron [6, 27]. No other signal was detected (data not shown).

### FTIR spectroscopy

FTIR difference spectroscopy was used to evaluate the impact of the mutations on the structural changes accompanying the oxidoreduction of iron center II. The reduced minus oxidized FTIR difference spectra recorded with the wild type and the I118S SOR mutant are superimposed in Fig. 2, spectra a. The spectra are very similar, notably below 1,450 cm−1, and the IR signatures of the histidine ligands (1,109 cm−1 for reduced SOR and 1,097 cm−1 for oxidized SOR) [40] were not significantly altered in the mutant. No IR band could be assigned to the side chain of S118, indicating that the redox state of the iron does not impact the vibrational properties of the S118 side chain. Significant differences with the wild type were revealed in the 1,450–1,700-cm−1 region for the I118S mutant. These differences are evidenced in the mutant minus wild type difference spectrum recorded in H₂O (Fig. 2, spectrum b). They consist in signals appearing at 1,670–1,660 (+)/1,683, 1,639 (−) cm−1 and at 1,554, 1,504 (+)/1,543, 1,523 (−) cm−1, that could be confidently assigned to amide I [v(C=O)] and amide II [v(CN) + δ(NH)] modes of peptide bonds, since they shifted by −3 to −11 cm−1 [v(C=O)] and by more than −50 cm−1 [v(CN) + δ(NH)], respectively, in spectra recorded in D₂O (Fig. 2, spectrum c). The bands observed between 1,630 and 1,680 cm−1 could result from frequency shifts of the v(C=O) mode of peptide groups in the mutant, as compared with the wild type. The I118A mutation induced very similar changes in the FTIR difference spectrum, associated with the reduction of iron center II, notably in the amide II region where the v(CN) + δ(NH) modes contribute, whereas only some of these changes were observed for the I118D mutation (Fig. S3). These data show that the I118S and I118A mutations have no major global impact on the structure of the SOR active site, but that they significantly affect the

### Potentiometric titrations

Potentiometric titrations of the I118 SOR mutants were monitored by electronic absorption spectroscopy at pH 7.6. Iron center II of each mutant was oxidized by addition of a stoichiometric amount of K₂IrCl₆, and the redox dependence of its absorption spectrum was studied by successive additions of sodium dithionite (Fig. S2). The reversibility of the redox process was checked by reoxidizing the fully reduced protein by addition of K₂IrCl₆, and was observed in all instances (data not shown). As shown in Table 1, the I118A and I118D mutations had no significant effect on the redox potential of center II at pH 7.6, whereas the I118S mutation increased it slightly.

### Table 1 Effects of the I118 superoxide reductase (SOR) mutations on the physicochemical properties of the SOR active site

| SOR protein    | Maximum of the absorption band of ferric iron center II (nm)a,b | Apparent pKₐ of the Fe⁵⁺–E47/Fe³⁺–OH transition | Redox potential of iron center II (mV, vs NHE) |
|----------------|---------------------------------------------------------------|---------------------------------------------|---------------------------------------------|
| Wild type      | 644 (1.90)                                                   | 9.0 ± 0.1                                   | 308 ± 11                                    |
| I118A          | 651 (1.90)                                                   | 9.3 ± 0.1                                   | 293 ± 7                                     |
| I118S          | 651 (1.92)                                                   | 9.6 ± 0.1                                   | 334 ± 12                                    |
| I118D          | 648 (1.92)                                                   | 9.2 ± 0.1                                   | 292 ± 7                                     |

NHE normal hydrogen electrode

a Observed at pH values lower than 8 and corresponding to the Fe³⁺–E47 species [16]

b The extinction coefficient (mM⁻¹ cm⁻¹) is given in parentheses

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IR mode frequencies of at least one peptide bond around iron center II.

Resonance Raman spectroscopy

Figure 3a shows the 15 K resonance Raman spectra of the K$_2$IrCl$_6$-oxidized wild type and I118 SOR mutants, excited at 647.1 nm, which allowed a resonance with the S$^-$(Cys)$\rightarrow$Fe$^{3+}$ charge transfer (CT) transition (644–651 nm). The resonance Raman spectra of SORs excited in such a condition show a number of bands arising from Fe–S stretch coupled with vibrations of the cysteine residue [27, 41]. This effect is very similar to that observed for blue copper proteins and Fe–S proteins for which the frequency, number, and relative intensity of the observed resonance Raman bands are dependent on the Fe–S bond strength and the conformation of the cysteine side chain [42–46].

The observed resonance Raman frequencies of wild-type D. baarsii and its I118 mutants are listed in Table 2.

Fig. 2 Reduced minus oxidized FTIR difference spectra of the SOR active site of the wild type (black line) and the I118 SOR mutant (red line) from D. baarsii (a). Potentials of 250 and 650 mV (versus the normal hydrogen electrode) were applied at the working electrode to obtain the reduced and oxidized states, respectively. The spectra consist of an average of data recorded on 30 electrochemical cycles, 300 scans per cycle, 4-cm$^{-1}$ resolution. Mutant minus wild type difference spectra calculated from the I118 SOR mutant in H$_2$O (b) or in D$_2$O (c).

Fig. 3 a 200–800 cm$^{-1}$ regions of 15 K resonance Raman spectra of the K$_2$IrCl$_6$ oxidized wild type and I118 SOR mutants, excited at 647.1 nm. For each spectrum, the contributions of the ice and the laser line at 669 cm$^{-1}$ were subtracted. The presentation of the resonance Raman spectra was done using the 742–744-cm$^{-1}$ band as an intensity standard. b Difference spectra (intensity multiplied by a factor of 2 with respect to the resonance Raman spectra in a). WT wild type
Table 2  Observed frequencies (cm$^{-1}$) of the resonance Raman bands of K$_2$IrCl$_6$-oxidized D. baarsii SOR and its I118 mutants, excited at 647.1 nm

| Wild type | I118S | I118A | I118D | Assignment |
|-----------|-------|-------|-------|------------|
| 218       | 219   | 218   | 220   | $\nu$(FeN(His)) |
| 241       | 241   | 240   | 240   | $\nu$(FeN(His)) |
| 277       | 279   | 280   | 279   |            |
| 287       | 289   | 287   | 288   |            |
| 299       | 302   | 302   | 302   | $\nu$(FeS) + $\delta$(Cys) |
| 306       | 308   | 309   | 308   | $\delta$(SC$_\beta$C$_a$) + $\nu$(FeS) |
| 317       | 314   | 318   | 316   |            |
| 324       | 325   | 325   | 324   |            |
| 344       | 349   | 346   | 345   |            |
| 358       | 359   | 356   | 356   | $\delta$(C$_3$C$_b$(O)) |
| 381       | 380   | 377   | 380   | + $\delta$(C$_3$C$_b$N) |
| 396       | 396   | 395   | 396   | + $\delta$(C(O)C$_2$N) |
| 402       | 404   | 406   | 403   | + $\delta$(C$_2$NC(O)) |
| 435       | 439   | 440   | 438   | + $\delta$(SC$_b$C$_a$) |
| 455       | 457   | 454   | 454   | + $\nu$(FeS) |
| 464       | 463   | 463   | 465   |            |
| 468       | 470   | 469   |       |            |
| 507       | 512   | 512   |       |            |
| 602       | 599   | 601   | 596   |            |
| 613       | 612   | 612   |       |            |
| 649       | 650   | 647   | 648   | $\delta$(His) |
| 658       | 659   | 658   | 658   | $\nu$(C$_b$N) |
| 665       | 665   | 665   | 665   |            |
| 703       | 703   | 702   | 701   |            |
| 713       | 714   | 713   | 712   |            |
| 742       | 744   | 744   | 743   | $\nu$(SC$_\beta$) |
| 765       | 764   | 764   | 763   |            |
| 777       | 777   | 777   | 777   |            |
| 793       | 795   | 793   | 793   |            |

The band assignments are from Clay et al. [27]

mainly corresponding to the S–C$\beta$ stretch [$\nu$(SC$_\beta$)] was associated with the 743-cm$^{-1}$ band [27]. Since the SORs from D. baarsii and D. vulgaris belong to the same 2Fe–SOR family and exhibit very similar spectroscopic properties [27], we analyzed our resonance Raman data on the basis of the band assignments proposed for the D. vulgaris enzyme [27].

The 299-cm$^{-1}$ band of the wild-type SOR from D. baarsii was significantly shifted to 302 cm$^{-1}$ in the spectra of the I118A, I118D, and I118S mutants (Figs. 3a and S4, Table 2). These upshifts were also clearly identified in the wild type minus I118 mutant difference spectra (Fig. 3b). When the intensity of the mode involving a major contribution of $\nu$(SC$_\beta$) is kept constant (742–744 cm$^{-1}$), one can observe an increase in the relative intensity of the 302-cm$^{-1}$ band of the mutants when compared with the 299-cm$^{-1}$ line of the wild-type SOR (Fig. 3a). In the resonance Raman spectra of blue copper systems and Fe–S proteins excited in S(Cys)-to-metal CT transitions, the most intense peak is generally the one exhibiting the largest $^{34}$S isotope sensitivity and thus having the predominant $\nu$(metal–S) character [42–46]. The same observations hold for the red-excited resonance Raman spectra of 1Fe-SORs and 2Fe-SORs [23, 27, 41]. Displacement of atoms along the Fe–S coordinate in the electronic excited state, involving the S$^-$ → Fe$^{3+}$ CT, is responsible for an Fe–S stretch being the most intense feature in the resonance Raman spectra resulting from excitation at 647.1 nm. The most intense line of the resonance Raman spectra of 2Fe-SORs is observed in the 290–310-cm$^{-1}$ region and exhibits the highest $^{34}$S sensitivity. The relative increased intensities of the 302-cm$^{-1}$ bands of the I118 mutants may be assigned to an increased resonance condition given the redshifts of the CT band (Table 1). Thus, the most intense resonance Raman band in the 300-cm$^{-1}$ region is a sensitive indicator of the Fe–S bond strength [23]. The upshifts from 299 cm$^{-1}$ for the wild-type enzyme to 302 cm$^{-1}$ for the three I118 mutants support an increased strength of the axial Fe–S(Cys) bond in the mutants (Table 2).

The 314–317-cm$^{-1}$ band of the wild-type SOR originates from kinematic and vibronic coupling of the internal cysteine deformations [$\delta$(SC$_b$C$_a$)] with the Fe–S stretch [27]. Owing to the strong decrease in resonance Raman activity of the 317-cm$^{-1}$ band, its position in the spectra of the I118 mutants is difficult to establish (Fig. 3a). The spectral deconvolutions indicate that its homolog corresponds to either the weak band at 314–316 cm$^{-1}$ or the weak or medium bands at 308–309 cm$^{-1}$ (Fig. S4). This decreased resonance Raman activity is hardly compatible with the redshifts of the S$^-$ (Cys) → Fe$^{3+}$ CT (Table 1). It would rather correspond to a change in coupling of the $\delta$(SC$_b$C$_a$) and $\nu$(Fe–S) modes, i.e., a decreased contribution of the Fe–S stretch in the normal mode composition of the 308–317-cm$^{-1}$ band. In the resonance Raman spectra of blue copper proteins and their model compounds, such a change in mode coupling originates from a modification of the (metal–S–C$\gamma$)–(S–C$_\beta$–C$_\alpha$) dihedral angle [43]. The resonance Raman bands in the 340–410-cm$^{-1}$ region were also significantly affected in the spectra of the I118 mutants (Figs. 3 and S4). These deformation modes of the cysteine ligand with the peptide backbone to which it is attached [27, 41] (Table 2) exhibit changes in frequency and/or in intensity, supporting some local protein reorganization. The spectral changes of the 310–410-cm$^{-1}$ resonance Raman bands are thus all consistent with a conformational change of the cysteine side chain and the peptide backbone in its proximity.

In the high-frequency region of the resonance Raman spectra, the 742-cm$^{-1}$ band of the wild-type SOR is gradually upshifted to 743 cm$^{-1}$ for the I118D mutant and to 744 cm$^{-1}$ for the I118A and I118S mutants (Fig. 3a). In
the difference spectra (Fig. 3b), the S-shaped signal in the 710–760-cm\(^{-1}\) region illustrates the marked sensitivity of the \(\nu(\text{SC})\) mode to the I118A and I118S mutations. Its increased frequency reflects an increase of the electron density of the S–C\(_{\beta}\) bond.

For the I118 SOR mutants, changes in the energy and the intensity of the resonance Raman modes involving the Fe–S–C\(_{\beta}\)H\(_{2}\)– bonds were observed. These changes are consistent with a conformational change of the cysteine side chain inducing a change in hydrogen bonding. On the basis of the frequency upshifts of the primarily \(\nu(\text{Fe–S(Cys)})\) and \(\nu(\text{S–C})\) modes, the Fe–S and S–C\(_{\beta}\) bond strengths are increased, indicating a decreased hydrogen-bonding state of the S(Cys) site.

Weak bands in the 210–240-cm\(^{-1}\) region have been attributed to stretching modes of the Fe–N(His) bonds \([\nu(\text{FeN(His)})]\) [27, 41]. Although in the I118 SOR mutants, intensity variations were detected for these bands, no significant frequency changes were observed (218–220 cm\(^{-1}\) and 240–241 cm\(^{-1}\)) (Fig. 3; Table 2). Thus, the equatorial Fe–N\(_4\)(His) coordinate is not significantly affected by mutations of the I118 residue.

Pulse radiolysis study of the I118 SOR mutants

The reaction of the I118 SOR mutants with O\(_2\)\(^{2-}\) was studied by pulse radiolysis, which allowed the observation of transient intermediates formed during the course of the reaction [18, 19]. The kinetics of the reaction were followed spectrophotometrically between 450 and 700 nm, and the protein was present in a large excess with respect to O\(_2\)\(^{2-}\), ensuring pseudo-first-order conditions. A 425-nm cutoff filter was positioned in the light beam to prevent photochemical reactions involving the reaction intermediates [18]. Similarly to the wild-type protein [16, 18, 19], all the I118 SOR mutants reacted very rapidly with O\(_2\)\(^{2-}\) to form, 50 \(\mu\)s after the pulse, the first reaction intermediate T1, proposed in the case of the \(D.\ baarsii\) enzyme to be an Fe\(^{2+}\)–superoxo species (Scheme 1) [19]. The absorption spectrum of T1 was not significantly affected by the I118 mutations, exhibiting a broad band centered at 600–610 nm, with an extinction coefficient of about 2.5 mM\(^{-1}\) cm\(^{-1}\) (Fig. S5). The second-order rate constants for the formation of T1 (\(k_1\)) were determined to be \((1.2 \pm 0.2) \times 10^9\), \((1.1 \pm 0.3) \times 10^9\), and \((1.4 \pm 0.3) \times 10^9\) M\(^{-1}\) s\(^{-1}\) for the I118A, I118D, and I118S SOR mutants, respectively. These values are very similar to the value determined for the wild-type protein \((1.0 \pm 0.2) \times 10^9\) M\(^{-1}\) s\(^{-1}\) [16, 19]. For the three mutants, \(k_1\) was pH-independent between pH 5 and pH 9.5 (data not shown), as reported for the wild-type protein [16, 19].

For the wild-type protein, the decay of T1 led to the formation of an SOR Fe\(^{3+}\)–OH/H\(_2\)O species (P1 in Scheme 1), with rate constant \(k_2\). Log \(k_2\) decreased linearly with increasing pH for pH < 8.0 and was pH-independent at pH ≥ 8, with a transition at pH 8. At pH < 7, the I118A, I118D, and I118S SOR mutations did not markedly affect the values of \(k_2\) and its pH dependence (Figs. 4 and S6). However, for the I118 mutants at pH > 7.0, the pH transition was lowered to pH 7 and \(k_2\) became pH-independent. Thus, the pH transition appeared at a more acidic pH in the mutants than in the wild-type SOR (Figs. 4 and S6). Consequently, the \(k_2\) values at pH > 8 were increased by a factor of about 4 for the I118 SOR mutants in comparison with the \(k_2\) value of the wild-type SOR.

The spectra of P1 for the I118 SOR mutants were reconstructed at pH 6.0 and pH 9.5, 10 and 180 ms after the beginning of the reaction, respectively (Figs. 5 and S7). For the wild-type SOR, the spectrum depends on the pH, reflecting the acid–base equilibrium between the Fe\(^{3+}\)–OH and Fe\(^{3+}\)–OH\(_2\) species (maximum absorbance at 560 and 640 nm, respectively), with an estimated pK\(_a\) of 7.0 [19] (Figs. 5 and S7). At pH 6.0, for the I118A, I118D, and I118S SOR mutants, the P1 spectra were almost superimposable on the spectrum of the wild-type SOR at the same pH (data not shown). However, for the I118A, I118D, and I118S SOR mutants at pH 9.5, the spectra differed from the spectrum of the wild-type SOR at the same pH, exhibiting a broad band with a maximum at 600–625 nm. Such a broad absorption band is consistent with the presence of a mixture of Fe\(^{3+}\)–Glu (644 nm) and Fe\(^{3+}\)–OH species (560 nm) at pH 9.5, which was similarly observed for the wild-type protein, but at a lower pH (pH 8.5) [19]. These data suggest that the pK\(_a\) of P1 in the I118 mutants was increased compared with that of the wild-type SOR.
For the wild-type SOR, the evolution of P1 to the final reaction product P2 (rate constant \(k_3\)) was shown to correspond to the binding of the E47 carboxylate side chain to the ferric iron site, in place of the OH/OH\(_2\) ligand (Scheme 1) [19, 38]. The pH dependence of \(k_3\) was proposed to reflect a general acid catalysis, associated with the \(pK_a\) of the \(\text{Fe}^{3+}\text{–OH}/\text{Fe}^{3+}\text{–OH}_2\) equilibrium (P1), where the aquo ligand was more easily displaced by the carboxylate side chain of E47 than the hydroxo ligand [17, 19]. For the I118A, I118D, and I118S SOR mutants, the pH dependence of \(k_3\) was still consistent with such a general acid catalysis, with, however, an apparent \(pK_a\) higher than that determined for the wild-type protein, 9.1 versus 7.0, respectively (Figs. 6 and S8). These data suggest that the I118A, I118D, and I118S SOR mutations induced a stabilization of the \(\text{Fe}^{3+}\text{–OH}_2\) species in the P1 species, in comparison with the wild-type SOR.

The spectrum of the final product, P2, was reconstructed at pH 6.0 and pH 9.5 for the I118 SOR mutants (Figs. 7 and S9). At pH 6.0, the mutants exhibited a spectrum similar to that of the wild-type protein, with an absorption band centered at 644 nm, associated with an \(\text{Fe}^{3+}\text{–Glu}\) species [16] (Figs. 7 and S9). At pH 9.5, the spectrum of the final product of the wild-type SOR exhibited an absorption band peaking at 560 nm, characteristic of an \(\text{Fe}^{3+}\text{–OH}\) species [16, 39]. For the I118A, I118D, and I118S SOR mutants at pH 9.5, the spectrum of the final product exhibited a broad absorption band with a maximum at 600–625 nm, consistent with the presence of a mixture of \(\text{Fe}^{3+}\text{–Glu}\) (644 nm) and \(\text{Fe}^{3+}\text{–OH}\) species (560 nm) at this pH. These results suggest an increase of the apparent \(pK_a\) of the final \(\text{Fe}^{3+}\text{–E47}\) species for these...
three SOR mutants, in comparison with that of the wild-type SOR. This is in agreement with the increase of the apparent pKₐ of the alkaline transition determined for these three mutants (Table 1).

Pulse radiolysis study of the E114A SOR mutant

When compared with the previous data obtained for the E114A SOR mutant [23], the I118A, I118D, and I118S mutations appeared to have opposite effects both on the strength of the Fe–S bond and on the protonation rate of the T1 intermediate, as observed by pulse radiolysis. However in the work reported in [23], the pulse radiolysis experiments were performed in conditions where photochemical reactions likely occurred at the level of the reaction intermediates [18]. Here, the reaction kinetics of the E114A mutant with O₂⁻ were reinvestigated in the presence of a 425-nm cutoff filter in order to prevent any photochemical processes [18]. Similarly to the wild type and the I118 SOR mutants, the reaction of the E114A mutant with O₂⁻ led to the formation of two transients T1 and P1, before evolving to the final reaction product P2 (data not shown). The rate constant of T1 formation (k₁) was not affected by the E114A mutation, and was found to be pH-independent between pH 5.5 and pH 9.5 (data not shown). Between pH 5.5 and pH 8.5, the decay of T1 (rate constant k₂) was directly proportional to the H⁺ concentration (Fig. S10a). However in this pH range, the k₂ values determined for the E114A mutant were six to nine times smaller than those determined for the wild-type SOR. At pH > 8.5, for the E114A mutant, k₂ increased slightly with decreasing H⁺ concentration, with values from seven to ten times smaller than those found for the wild-type SOR (Fig. S10a). These data show that the E114A SOR mutation decreases the rate of protonation of T1, as previously reported [23]. This indicates that the photochemical processes occurring in the former study did not affect k₂. As shown in Fig. S10b, the pH dependence of k₃ for the E114A SOR mutant exhibited an apparent pKₐ very similar to that of the wild-type SOR. However, the E114A mutation induced a decrease of the k₃ values by a factor of 2.5–10 between pH 5.5 and pH 9.6 (Fig. S10b).

Density functional theory calculations

To rationalize the effects of hydrogen bonds involving the sulfur ligand on the reactivity of SOR with O₂⁻, three models corresponding to the five-membered Fe²⁺ form, the T1 intermediate (Fe²⁺–O₂⁻), and its subsequently protonated species (Fe³⁺–OOH) were studied by density functional theory (DFT) calculations. A fourth structure, Fe³⁺–OH, was also considered, to account for a model of the ferric state of SOR after formation of hydrogen peroxide. Each intermediate was modeled by three models, each with a different number of N/C0H/C1/C1/S interactions. Model A has no hydrogen bond involving the sulfur ligand, model B has one hydrogen bond, and model C has two hydrogen bonds (wild-type SOR). Figure 8 shows the structure of models A, B, and C for the Fe²⁺–O₂⁻ species (T1). The geometric parameters (Fe–S and Fe–O distances) as well as the charges and spin populations of the iron and sulfur atoms are presented in Table 3 for the four species Fe²⁺, Fe²⁺–O₂⁻, Fe³⁺–OOH, and Fe³⁺–OH, each described by models A, B, and C. A more complete set of data is available in Tables S1, S2, and S3.

![Fig. 8 Optimized structures of models A, B, and C of the active site of SOR, binding superoxide anion (Fe²⁺–O₂⁻ species). Model C includes the two hydrogen bonds involving the sulfur ligand, as found in wild-type SOR. Model B has only one hydrogen bond and model A has no hydrogen bond. Relevant atoms are indicated in model C. The hydrogen bonds considered are represented by dashed green lines. Circled atoms were kept fixed during geometry optimization, conducted at the B3LYP/B1 level of theory.](image-url)
For the five-membered Fe$^{2+}$ structure, models A, B, and C showed good agreement with the corresponding PDB structure of SOR (2IJ1) [14]. In terms of geometry, the Fe–S bond is slightly affected by the number of hydrogen bonds involving sulfur, having a distance of 2.35 Å in model A, 2.37 Å in model B, and 2.39 Å in model C (2.41 Å in PDB). Consistently, a slight increase of the atomic charge and spin population of iron, accompanied by a decrease of the respective values for sulfur, is found when one and then two hydrogen bonds involving sulfur are included. These trends are significant and indicate that the Fe–S bond is weakened by hydrogen bonds involving sulfur. Similar observations were made for the Fe$^{2+}$–O$_2^{•–}$ and Fe$^{3+}$–OOH species.

As for the five-coordinate Fe$^{2+}$–O$_2^{•–}$ species, comparison of structures for models A, B, and C shows that the Fe–S bond is lengthened if the number of hydrogen bonds is increased. This trend is noticeable, since the Fe–S distance is 2.55 Å in model A, 2.61 Å in model B, and 2.72 Å in model C. Consistently, the Fe–O distance decreases with the increase of the Fe–S distance: the Fe–O bond lengths are 2.29 Å in model A, 2.23 Å in model B, and 2.20 Å in model C. The presence of hydrogen bonds thus modulates the trans influence between the sulfur ligand and the superoxide.

Protonation of the distal oxygen of the Fe$^{2+}$–O$_2^{•–}$ species leads to the iron–hydroperoxo intermediate (Fe$^{3+}$–OOH), where the hydroperoxide moiety has an overall charge of −1.1, with a spin population of about 0.3 in the models A, B, and C. The substrate protonation is accompanied by an increase of the atomic charge of the iron in the three models (e.g., in model C, the charge of iron is 1.34 for the Fe$^{2+}$–O$_2^{•–}$ species and 1.52 in the Fe$^{3+}$–OOH species). Accordingly (see Table 3), the sulfur ligand becomes less negative in the three models after protonation. The spin populations of the iron and sulfur atoms also increase during this process. This shows that the substrate moiety loses its radical character by abstracting one electron after protonation, thus leading to a formal Fe$^{3+}$ covalently bound to an OOH$^{•–}$ moiety. In terms of geometry, the Fe–S and Fe–O bonds are shorter than in the Fe$^{2+}$–O$_2^{•–}$ structure. This is consistent with an iron atom formally losing one electron.

When models A, B, and C for the Fe$^{3+}$–OOH species are compared, the general trends observed in the previous Fe$^{2+}$–O$_2^{•–}$ intermediates are found again. The presence of hydrogen bonds involving sulfur makes the Fe–S bond longer and the Fe–O bond shorter. However, this effect remains much more limited than for the Fe$^{2+}$–O$_2^{•–}$ species. For the Fe$^{3+}$–OOH species, the changes in Fe–S and Fe–O bond lengths in the range of 0.04–0.05 Å in the three models. Although hydrogen bonds have a limited effect on the geometries, their influence on the electronic structure is more important. For example, the spin population of sulfur is larger in model A than in models B and C, with respective values of 0.49, 0.38, and 0.33. Consistently, the charge separation between sulfur and iron is enhanced by the presence of hydrogen bonds (sulfur becomes more negative and iron becomes more positive; see the charges in Table 3). As for the presence of hydrogen bonds weakens the Fe–S interaction, which in turn strengthens the Fe–O interaction. Indeed, the charge of the oxygen atom bound

### Table 3: Results of density functional theory calculations for models A, B, and C, with relevant distances (Fe–S and Fe–O), charges ($q$), and spin populations ($S$) of iron and sulfur atoms.

|           | Fe$^{2+}$       | Fe$^{2+}$–O$_2^{•–}$ | Fe$^{3+}$–OOH | Fe$^{3+}$–OH |
|-----------|----------------|----------------------|----------------|--------------|
|           | A   | B   | C   | A   | B   | C   | A   | B   | C   | A   | B   | C   |
| Fe–S (Å)  | 2.35 | 2.37 | 2.39 | 2.55 | 2.61 | 2.71 | 2.47 | 2.49 | 2.52 | 2.50 | 2.54 | 2.58 |
| Fe–O$_p$ (Å) | 1.26 | 1.29 | 1.32 | 1.30 | 1.32 | 1.34 | 1.46 | 1.50 | 1.52 | 1.51 | 1.56 | 1.58 |
| $q_{Fe}$   | 3.49 | 3.51 | 3.53 | 3.51 | 3.52 | 3.53 | 3.69 | 3.72 | 3.74 | 3.74 | 3.77 | 3.79 |
| $S_{Fe}$   | −0.49 | −0.50 | −0.54 | −0.58 | −0.57 | −0.60 | −0.29 | −0.37 | −0.42 | −0.34 | −0.41 | −0.46 |
| $S_{S}$    | 0.23 | 0.20 | 0.18 | 0.13 | 0.11 | 0.08 | 0.49 | 0.38 | 0.33 | 0.42 | 0.32 | 0.28 |

Values are given for the four intermediates studied with no (model A), one (model B), and two (model C) hydrogen bonds involving sulfur. Geometries were obtained at the B3LYP/B1 level of theory, and charges and spin populations are calculated at the B3LYP/B2//B3LYP/B1 level of theory.
to iron decreases and its spin population increases on going from model A to models B and C. As can be seen in Table 3, the last structure studied, the Fe$^{3+}$–OH species which is formed after the release of H$_2$O$_2$, shows trends similar to those already mentioned. The Fe–S bond is shortened and the Fe–O bond becomes longer when hydrogen bonds involving sulfur are added. The effect of hydrogen bonding is of comparable magnitude as previously noted, with a variation of +0.08 Å for Fe–S and −0.05 Å for Fe–O between models A and C. The charges and spin populations are also in agreement with a reinforced Fe–S interaction when hydrogen bonds are absent. These observations are in agreement with the increased strength of the Fe–S bond in the I118 SOR mutants observed by resonance Raman spectroscopy (Fig. 3). Resonance Raman spectroscopy also showed an increase of the C$_\beta$–S interaction. However, owing to the constraints applied to our models (fixed C$_\gamma$), our calculations did not provide any sizeable modification of the C$_\beta$–S distance, which remains at values close to 1.86 Å in the models A, B, and C of the Fe$^{3+}$–OH resting state.

Finally, we also studied the energetics for the protonation process of the Fe$^{2+}$–O$_2$•$^-$ intermediate to form the Fe$^{3+}$–OOH species, which was computed for models A, B, and C. For models A, B, and C, the protonation process was found to be exothermic by 41.2, 36.2, and 32.6 kcal mol$^{-1}$, respectively, with the use of −260 kcal mol$^{-1}$ as a reference for the solvated proton [37] and B3LYP/B2/B3LYP/B1 plus solvation effects for calculated values. These values are consistent with the −36 kcal mol$^{-1}$ reported by Dey et al. [30] for the same reaction, using a similar approach. Comparison of the values found for the three models shows that hydrogen bonds involving the sulfur atom make the protonation process less exothermic. Compared with model C (wild-type SOR), the reaction is more exothermic in model B (I118 SOR mutants) by 5.6 kcal mol$^{-1}$ and in model A (no hydrogen bonds) by 8.9 kcal mol$^{-1}$. Hence, thermodynamically, the presence of hydrogen bonds disfavors the protonation process of the first reaction intermediate T1. These data are in agreement with the experimental observations presented in Fig. 4, showing that the I118 SOR mutations, which impaired one hydrogen bond involving the sulfur ligand, induced an increase of the protonation rate of the T1 intermediate.

Discussion

The presence of hydrogen bonds involving the sulfur atom of cysteine ligands is prominent in iron proteins, e.g., in heme–thiolate proteins such as cytochrome P450 or [Fe–S] cluster proteins [24–26]. In SORs, two peptide NH groups from L/I118 and H119 are within hydrogen-bonding distance of the C116 axial sulfur ligand (Fig. 1) [11–14], and they have been proposed to play an important function in SOR [27, 30]. However, up to now, no experimental data were reported concerning their role in catalysis. Site-directed mutagenesis is a suitable technique for such investigations; however, some limitations have to be taken into account. As highlighted in “Introduction,” the imidazole side chain of H119 is a ligand of the iron and consequently this position cannot be mutated without introducing large perturbations, and possibly demetalation of the active site. For the buried I118 position, mutation into a proline residue to probe the function of its peptide NH group could also induce large conformational changes in the protein. In fact, in the different amino acid sequences of SOR available to date, no proline residue was found in this position. Here we mutated the I118 residue into alanine, aspartate, and serine, assuming that the modifications of the side chain of I118 could impact the orientation and the strength of the peptide NH hydrogen bond involving the sulfur ligand.

Such specific effects of the I118 mutations on the peptide NH hydrogen bond were supported by FTIR spectroscopy, a very sensitive technique to investigate the structural modifications accompanying the change in the redox state of the SOR iron site [22, 23]. FTIR spectroscopy showed that whereas the I118 mutations had no major impact on the active-site structure, they significantly affected the conformation of at least one peptide bond around the iron active site, which is compatible with a change in the hydrogen bond involving one or more peptide NH groups.

Resonance Raman spectroscopy further showed that the I118A, I118D, and I118S mutations had very specific effects on the cysteine ligand. For these three mutants, the frequencies of the stretching modes involving the Fe–S–C$_\gamma$ side chain of H119 is a ligand of the iron and consecutively this position cannot be mutated without introducing large perturbations, and possibly demetalation of the active site. For the buried I118 position, mutation into a proline residue to probe the function of its peptide NH group could also induce large conformational changes in the protein. In fact, in the different amino acid sequences of SOR available to date, no proline residue was found in this position. Here we mutated the I118 residue into alanine, aspartate, and serine, assuming that the modifications of the side chain of I118 could impact the orientation and the strength of the peptide NH hydrogen bond involving the sulfur ligand.

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spin \((S = 5/2)\) with a rhombic geometry \((E/D = 0.33)\), as reported for the wild-type SOR from \(D. baarsii\) [6]. No axial feature, such as the features reported for the SOR from \(Pyrococcus furiosus\) [47] and \(Treponema pallidum\) [48], was found in the EPR spectra of the I118 SOR mutants.

Taken together, these data demonstrated that the I118A, I118S, and I118D mutations have a similar effect on the SOR active site. They all induced an apparently similar decrease in the strength of the hydrogen bond between the peptide NH and the sulfur ligand, which in turn led to a strengthening of the \(\text{Fe}^{3+}-\text{S(Cys)}\) bond. This effect most likely originates from a decrease in the size of the side chain of residue 118 that could slightly remodel the structure of the C116–N117–I118–H119 tetrapeptide. This decrease in size has to be significant since we found no frequency change for the modes involving the \(\text{Fe}–\text{S(Cys)}\) bonds when we compared the resonance Raman spectrum of the wild-type SOR with that of the I118V mutant (data not shown).

Surprisingly, the strengthening of the \(\text{Fe}^{3+}-\text{S(Cys)}\) bond observed in the I118A, I118D, and I118S mutants has almost no effect on the redox potential of the iron active site. Only a small positive variation for the I118S mutant was observed (Table 1). The redox process occurring at the SOR active site is rather complex, involving both electron transfer and proton transfer and coordination of the carboxylate side chain of E47 to the ferric iron. Dey et al. [30] suggested by DFT calculations that in SOR hydrogen bonds involving the thiolate ligand could have an opposite effect on the oxidation of the ferrous iron and on the subsequent coordination of the E47 ligand to the ferric iron. In addition, the redox potential also depends on the stabilization and/or destabilization of the oxidized and/or reduced form of the redox center, and the I118 mutants could also strengthen the \(\text{Fe}^{2+}-\text{S(Cys)}\) (ferrous) bond. However, no information on the effect of the I118 SOR mutants on the \(\text{Fe}^{2+}-\text{S(Cys)}\) bond is available yet. The ferrous form does not exhibit a visible absorption band and could not be investigated by resonance Raman spectroscopy.

The I118 peptide NH hydrogen bond involving the sulfur ligand controls the \(pK_a\) of the reaction intermediates

The pulse radiolysis studies of the reactivity of SOR with \(\text{O}_2^{•−}\) further showed that the presence of the I118 peptide hydrogen bond involving the sulfur ligand has a direct effect on the \(pK_a\) of the different intermediates species that are formed \(\text{trans}\) to the \(\text{Fe}–\text{S(Cys)}\) bond during catalysis. Whereas the initial fast binding of \(\text{O}_2^{•−}\) to the ferrous iron site to form T1 \((k_1)\), was not significantly modified by the I118A, I118D, and I118S mutations, they all induced an increase in the rate of protonation of T1 \((\text{Fe}^{2+}\text{–O}_2^{•−}\) species) at pH > 8.0, where \(\text{H}_2\text{O}\) was proposed to be the proton donor [19]. Since I118 is located on the opposite side of the iron atom compared with the \(\text{O}_2^{•−}\) binding site (Figs. 1 and 8), and since no major structural changes were observed in the mutants by FTIR spectroscopy, the increase in the rate of T1 protonation could hardly be explained by any structural modifications that might facilitate proton transfer from the water molecule to the superoxo moiety of T1. Rather, the faster rate of protonation of T1 could be better associated with a specific increase of its \(pK_a\). This is in agreement with the Brønsted catalysis equation, which predicts that among a series of bases, the logarithm of the rate constant of protonation of a species is directly proportional to its \(pK_a\). Such modification of the \(pK_a\) of T1 could result, through a \(\text{trans}\) electronic effect, from the strengthening of the \(\text{Fe}–\text{S(Cys)}\) bond in the I118 mutants.

At pH < 8, where \(\text{H}_2\text{O}^{+}\) was shown to be the proton donor [19], the I118 mutations did not affect the rate of protonation of T1. This could be in line with the fact that in the presence of a strong acid, the dependence of the rate of protonation of a weak base (T1) on its \(pK_a\) might not be valid any more.

Such an effect of the I118 peptide NH hydrogen bond involving the sulfur ligand on the \(pK_a\) of the T1 intermediate was further supported by DFT calculations (Fig. 8; Table 3). The calculations showed that for the \(\text{Fe}^{2+}\text{–O}_2^{•−}\) species (T1), the \(\text{Fe}–\text{S(Cys)}\) distance increased significantly in the presence of hydrogen bonds involving the sulfur ligand, whereas the \(\text{Fe}–\text{O}\) distance decreased slightly. The presence of hydrogen bonds rendered the protonation of the \(\text{Fe}^{2+}\text{–O}_2^{•−}\) species less exothermic, by 5.6 and 8.9 kcal mol\(^{-1}\) in the presence of one and two hydrogen bonds, respectively. The Bell–Evans–Polanyi principle [49, 50] stipulates that for similar reactions, the most exothermic one will have the lowest activation energy. Thus, one can anticipate a lowering of the activation energy of protonation of the \(\text{Fe}^{2+}\text{–O}_2^{•−}\) species in the presence of only one of the two hydrogen bonds, as is the case for the I118 SOR mutants. These computational data are in full agreement with the experimental observations, which showed that the reaction rate \(k_2\) for the protonation of the first reaction intermediate was increased in the I118 mutants compared with wild-type SOR. These data also suggest that the second hydrogen bond involving the sulfur ligand and the H119 NH amide group should have a similar effect on \(k_2\). Thus, the two hydrogen bonds involving I118 and H119 and the sulfur ligand should have an additive impact on the \(pK_a\) of T1.

A similar effect of the presence of a hydrogen bond involving the sulfur ligand was also observed by pulse radiolysis on the transient P1, formed following the release

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of H2O2 from the active site and associated with Fe3+-OH/Fe3+-OH2 species in acid–base equilibrium [19]. The data showed that the I118A, I118D, and I118S mutations induced an increase of the apparent pKα of P1 by two pH units (from 7.0 to 9.1; Figs. 6 and S8), suggesting that removal or weakening of a hydrogen bond involving the sulfur ligand in the I118 mutants induces a stabilization of the Fe3+-OH2 species compared with the Fe3+-OH species. Again, and similarly to what was described for the Fe3+–O22− species, this supports a trans electronic effect of the sulfur ligand on the pKα of the Fe3+-OH/Fe3+-OH2 species, which is modulated by the hydrogen bond involving this ligand.

Hence, both theoretical and experimental observations support the hypothesis that the presence of hydrogen bonds involving the sulfur ligand of the active site of SOR disfavors the protonation of both the superoxide adduct of the Fe3+–O22− intermediate and the OH adduct of the Fe3+-OH species, as observed in the different X-ray structures of this intermediate trapped in an SOR crystal [14]. Any impairment of this specific protonation process due to a bad positioning of the K48 side chain might promote the formation of an iron–oxo species, as supported by our studies on the K48I SOR mutant [19]. The ability of the I118 SOR mutants to favor the formation of an iron–oxo species in their active sites is currently under investigation.

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