Superoxide reduction by a superoxide reductase lacking the highly conserved lysine residue

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6 **Abbreviations:** ROS, reactive oxygen species; SOD, superoxide dismutase; SOR,
7 superoxide reductase; $\langle \mathbf{V} \rangle_{\text{Fe-S}}$ - intensity weighted frequencies

8

Abstract

Superoxide reductases (SORs) are the most recently identified superoxide detoxification systems, being found in microorganisms from the three domains of life. These enzymes are characterized by a catalytic mononuclear iron site, with one cysteine and four histidine ligands of the ferrous active form. A lysine residue in the –EKHVP– motif, located close to the active site, has been considered to be essential for the enzyme function, by contributing to the positive surface patch that attracts the superoxide anion and by controlling the chemistry of the catalytic mechanism through a hydrogen bond network. However, we show here that this residue is substituted by non-equivalent amino acids in several putative SORs from Archaea and unicellular Eukarya. In this work, we focus on mechanistic and spectroscopic studies of one of these less common enzymes, the SOR from the hyperthermophilic crenarchaeon *Ignicoccus hospitalis*. We employ pulse radiolysis fast kinetics and spectroscopic approaches to study the wild type enzyme (-E₂₃T₂₄HVP-), and two mutants, T24K and E23A, the later mimicking enzymes lacking both the lysine and glutamate (a ferric ion ligand) of the motif. The efficiency of the wild type protein and mutants in reducing superoxide is comparable to other SORs, revealing the robustness of these enzymes to single mutations.

1 Introduction

2 Dioxygen can be activated by univalent reduction to the superoxide anion radical
 3 ($O_2^{\cdot -}$), which upon further reaction with transition metals or radicals (e.g. semiquinones)
 4 leads to the formation of reactive oxygen species (ROS), such as the superoxide anion.
 5 Most organisms have systems to prevent the harmful effects caused by superoxide of
 6 derived species, the superoxide dismutases and the superoxide reductases (SORs)
 7 (recently reviewed in (Kurtz, 2006, Pinto *et al.*, 2010, Sheng *et al.*, 2014),

8 SORs are mononuclear iron enzymes that can be classified in two classes according to
 9 the number of iron centers (Kurtz, 2006, Pinto *et al.*, 2010, Sheng *et al.*, 2014) (not
 10 taking into consideration the recently discovered methanoferrodoxins) (Kratzer *et al.*,
 11 2011): 1Fe-SORs (or neelaredoxins (Chen *et al.*, 1994)) and 2Fe-SORs (or
 12 desulfoferrodoxins (Lucchetti-Miganeh *et al.*, 2011, Moura *et al.*, 1990)). SORs perform
 13 only the reductive process of the superoxide dismutation process (equation 1)

15 where the ferrous active form is regenerated through the action of electron donors
 16 (rubredoxins in some cases), that are themselves reduced by NAD(P)H
 17 oxidoreductases. The SORs are not restricted to anaerobes, being found in facultative
 18 prokaryotes and aerobic Eukaryotes (Lombard *et al.*, 2000, Lucchetti-Miganeh *et al.*,
 19 2011).

20 Superoxide reductases have as the active site where the reduction of $O_2^{\cdot -}$ takes place a
 21 pentacoordinated Fe^{2+} ion bound to four equatorial histidines and one axial cysteine in a
 22 square pyramidal geometry. In the oxidized enzyme, the sixth axial site is typically
 23 occupied by a glutamate, although in a subset of enzymes this residue is substituted by

1 a non metal-ligating amino acid, thus leaving the sixth position vacant or occupied by a
2 water/hydroxide molecule.

3 The catalytic mechanism involves iron-superoxide or peroxide intermediates, as
4 proposed on the basis of visible spectroscopy coupled to pulse radiolysis or stopped
5 flow techniques, and resonance Raman spectroscopy (Abreu *et al.*, 2000, Lombard *et*
6 *al.*, 2001, Lombard *et al.*, 2000, Mathé *et al.*, 2006, Nivière *et al.*, 2004, Nivière &
7 Lombard, 2002, Nivière *et al.*, 2001, Rodrigues *et al.*, 2006, Rodrigues *et al.*, 2007,
8 Rodrigues *et al.*, 2008, Bonnot *et al.*, 2012), as well as theoretical calculations (Bonnot
9 *et al.*, 2012, Silaghi-Dumitrescu *et al.*, 2003, Sit *et al.*, 2011, Dey *et al.*, 2007). Two
10 amino acid residues, Glu14 and Lys15 (*Pyrococcus furiosus* 1Fe-SOR numbering (Yeh
11 *et al.*, 2000)) (Fig. 1), present in the highly conserved -EKHVP- motif (where H is one of
12 the iron histidine ligands and E is the sixth ligand to the ferric iron in most enzymes)
13 have been considered catalytically important. The glutamate was suggested to be
14 involved in product release from the catalytic site or in proton transfer events. However,
15 site-directed mutagenesis studies in which the glutamate residue was replaced by non-
16 negative or neutral residues (alanine, valine or glutamine) (Abreu *et al.*, 2001, Emerson
17 *et al.*, 2002, Lombard *et al.*, 2001, Rodrigues *et al.*, 2006) and the study of a naturally
18 occurring 1Fe-SOR lacking this glutamate (Rodrigues *et al.*, 2008), revealed that the
19 catalytic rates of superoxide reduction are not modified upon its substitution. The
20 neighboring lysine residue contributes to a positive surface charge near the iron center
21 favoring the electrostatic attraction of the substrate. More importantly, this lysine was
22 proposed to play a key role in controlling the evolution of the iron superoxide or
23 peroxide ligand during catalysis, primarily on the basis of site-directed mutagenesis
24 studies of the *Desulfoarculus baarsii* 2Fe-SOR (Bonnot *et al.*, 2012, Lombard *et al.*,
25 2001): the rate constant of the first intermediate formation decreased by a factor of

almost 25 in this enzyme upon mutation of the lysine. In a similar study with the 2Fe-SOR from *Desulfovibrio vulgaris* a smaller effect was observed, decreasing the rate by only about 7 times (Emerson *et al.*, 2002). The role of the lysine was further strengthened by the determination of the three dimensional structure of peroxide intermediates of the *D. baarsii* enzyme incubated with hydrogen peroxide, which showed that the lysine side chain interacts with the peroxide moiety, possibly playing a role in proton transfer for hydrogen peroxide release (Katona *et al.*, 2007).

In this work, a search of the protein sequence data bases revealed the existence of several putative SORs with no lysine residues in the conserved motif, including the 1Fe-SOR from *Ignicoccus (I.) hospitalis*, in which the motif -EKHVP- is substituted by -E₂₃T₂₄H₂₆P- (*I. hospitalis* SOR numbering), i.e., the lysine is replaced by a threonine. *I. hospitalis* is a hyperthermophilic anaerobic Crenarchaeon growing at an optimum temperature of 90°C (Podar *et al.*, 2008). Putative enzymes lacking both the lysine and the glutamate residues were also found in genomes from Eukaryotes (Lucchetti-Miganeh *et al.*, 2011, Testa *et al.*, 2011). Here we address the role of the amino acid residues Glu14 and Lys15 in the catalytic cycle by undertaking a study of the wild type enzyme from *I. hospitalis* (herein referred to as *I. hospitalis* SOR) and two site-directed mutants, by fast kinetics and spectroscopic methods. The mutants were constructed in order to i) simulate enzymes that lack both the lysine and the glutamate residues and ii) “reconstruct” the canonical enzyme-like active site by substituting the threonine with a lysine. The results are discussed and compared with the data available in the literature for the canonical enzymes.

Materials and Methods

Gene cloning and protein production

Wild type *I. hospitalis* SOR was cloned into pET24(+) as previously reported (Pinho *et al.*, 2010). This plasmid gene was used as a template for site-directed mutagenesis to create the E23A and T24K mutants, using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene) and pairs of appropriate primers and for the E23A mutant, 5'-GCC ATA TCC AAG GCT **GCC** ACC CAT ACG CCC AAG A-3' and 5'-TCT TGG GCG TAT GGG TGG **CAG** CCT TGG ATA TGG C-3' as reverse and forward primers, respectively, and for the T24K mutant 5'- CCA AGG CTG AGA **AGC** ATA CGC CCA AGA -3' and 5'- TCT TGG GCG TAT GCT **TCT** CAG CCT TGG -3' as forward and reverse primers, respectively. The plasmids were sequenced to confirm the absence of undesirable mutations, and the presence of the introduced ones. All recombinant plasmids were then transferred to *E. coli* BL21(DE3) Gold cells, which were grown aerobically at 37 °C in M9 minimal medium supplemented with 30 µg mL⁻¹ kanamycin and 200 µM FeSO₄. At OD_{600nm}=0.3, 400 µM isopropyl-1-thio-β-D-galactopyranoside was added, the temperature was lowered to 28 °C and the growth continued for 20 h. The cells were afterwards harvested by centrifugation at 10 000 g for 10 min at 4 °C.

Purification

All three proteins were purified by the same procedures, as reported for the wild type enzyme (Pinho *et al.*, 2010). The protein purity was judged on the basis of SDS-PAGE (Laemmli, 1970) and the protein concentration and iron content were determined by the bicinchoninic acid protein assay (Pierce) (Smith *et al.*, 1985) and the 2,4,6- tripyridyl-s-triazine method (Fischer, 1964), respectively. The yield of pure protein was 17 mg for

the wild type protein and E23A mutant and 3 mg for the T24K mutant, per liter of growth.

Spectroscopic Studies

UV-Visible spectra were recorded at room temperature on a Shimadzu UV-1603 spectrophotometer. In resonance Raman experiments, back-scattered light from the frozen protein sample (77 K) was collected by a confocal microscope coupled to a spectrograph (Jobin Yvon, LabRam). About 2 μ L of 3 mM ferric protein was introduced into a liquid nitrogen cooled cold finger (Linkam THMS600) mounted on a microscope stage. The spectra were recorded with 40 s accumulation time and laser (λ_{exc} 647nm) power of 7-15 mW, provided by a Kr⁺ laser (Coherent Innova 300). After polynomial background subtraction, resonance Raman spectra (275 - 425 cm^{-1} region) were submitted to band fitting analysis by a 'home-made' program.

pH studies by Stopped-Flow spectrophotometry

The pKa of the oxidized form of *I. hospitalis* SORs was determined using a Bio-Logic stopped-flow SM300/S apparatus equipped with a TIDAS spectrometer. The change in pH was accomplished by mixing of 145 μ M of SOR (in 20mM Tris-HCl pH 7.6 plus 150mM NaCl), in one syringe, in a 1:5 ratio with the buffer (100mM of BisTris propane, 2-(N-morpholino)ethanesulfonic acid (MES) or N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) at the desired pH in the other syringe. The spectra were recorded and those acquired after the reaction was completed were used to analyze the pH induced wavelength shifts. The change in pH was confirmed by directly measuring the pH of a solution with a protein to buffer ratio of 1:5. This method was necessary since at high pH values the proteins start slowly degrading, as also observed previously for the *G. intestinalis* SOR (Testa *et al.*, 2011).

1 *Redox Titrations*

2 Redox titrations followed by UV/Visible spectroscopy were performed anaerobically in
3 50 mM Tris-HCl and 50 mM BisTris propane and MES (2-(*N*-morpholino)ethanesulfonic
4 acid), by a stepwise addition of sodium ascorbate as reductant or potassium
5 hexachloroiridate(IV) (K_2IrCl_6) as oxidant, as described in (Rodrigues *et al.*, 2008).
6 Experimental data were analyzed with Nernst equations for single one-electron
7 transitions and the reduction potentials are reported relative to the standard hydrogen
8 electrode.

9 *Reactivity towards Superoxide- Pulse Radiolysis assays*

10 Pulse radiolysis experiments were performed at Brookhaven National Laboratory (BNL)
11 using a 2 MeV Van de Graaff accelerator and the $(SCN)_2^-$ dosimeter as previously
12 described (Goto *et al.*, 1998). O_2^- (1-20 μ M) was generated using doses of 100-2500
13 rads. All pulse radiolysis samples were prepared in Millipore ultra-purified distilled water.
14 When needed, the protein was reduced with the addition of stoichiometric quantities of
15 sodium ascorbate. The experiments were carried out at 25°C and at 65°C in air-
16 saturated solution containing: i) 4 to 8 μ M SOR in 0.1 M phosphate, 50 mM formate, 10
17 μ M EDTA, pH 7.4, for superoxide dismutase activity measurements; ii) 40 to 100 μ M
18 SOR in 2 mM Tris-HCl, 6.6 mM NaCl and 50 mM formate or 0.5 M of ethanol for SOR
19 mechanistic studies. The oxidation of the SORs by O_2^- was measured at 500-700 nm
20 by following the absorbance changes as a function of time, at a microsecond to second
21 time scale. The data were analyzed with the BNL Pulse Radiolysis Program, Prwin. All
22 quoted rates are the average of at least three measurements and the cumulative error
23 in these values is approximately 10 percent.

24

Results and Discussion

Amino acid sequence

The SOR from the hyperthermophile *Ignicoccus hospitalis* was selected for this study after an extensive comparison of SOR amino acid sequences as the lysine of the highly conserved –EKHVP– motif proposed to be essential for catalysis is substituted by a threonine in this enzyme (T24 in *I. hospitalis* SOR amino acid numbering; unless otherwise stated, we will use throughout the remaining of the article this numbering). The amino acid sequence alignment shows that the five ligands (four histidines, one cysteine) coordinating the ferrous ion are all strictly conserved. However, twelve other 1Fe-SORs from Archaea, Bacteria and unicellular Eukaryotes have that lysine substituted by a serine, a threonine or a glycine (Fig. 1). Interestingly, some SORs (from the bacterium *Beggiatoa*, and the Eukarya *Monosiga brevicollis* and *Salpingoeca sp.*, see Fig. 1) lack both the glutamate ligand of the ferric form and the lysine, for which the *I. hospitalis* SOR E23A mutant will be a model. The amino acid sequence similarities and identities of *I. hospitalis* SOR to other prokaryotic SOR sequences are approximately 78% and 63%, respectively, for the lysine lacking SORs and ca. 44% and 34% for the remaining ones. These values are much lower (ca. 26% and 16%, Fig. 1) for the eukaryotic enzymes. The overall amino acid similarities and identities suggest that, unlike other residues considered relevant, the mutations of the lysine or glutamate occurred more than once upon evolution.

We first analyzed the biochemical, spectroscopic and thermodynamic properties of the catalytic center of the studied proteins and then proceeded to the study of their catalytic behavior.

1 *Protein Characterization*

2 The *I. hospitalis* SORs (wild type and the two mutants, E23A and T24K), were
3 successfully overexpressed in *E. coli* and isolated as homotetramers, as determined by
4 gel filtration. Each monomer revealed in SDS-PAGE a molecular mass of 14 kDa, in
5 agreement with the amino acid sequence. The iron content of each SOR indicated 0.95
6 ± 0.1 atoms of Fe per monomer, confirming a full occupancy of the active site. The ferric
7 and acidic forms of the *I. hospitalis* SORs have characteristic visible spectra with
8 maxima at 640 nm (wt, pH 7), 650 nm (T24K, pH 7) and 660 nm (E23A, pH 5), which
9 originate from the charge transfer transition involving the cysteine ligand and Fe^{3+} (Clay,
10 Jenney, Hagedoorn, *et al.*, 2002), and a shoulder at 340 nm. Upon pH increase (pH
11 above 10 for the wt and T24K mutant, pH above 7 for the E23A mutant), the charge
12 transfer band is blue-shifted to 550 nm (Fig. 2), while the 340 nm band bleaches. The
13 apparent pKa values for these transitions were determined by stopped flow experiments
14 (Fig. 3, Table 1): 10.5 and 10.7 for the wild type and T24K proteins, respectively, and
15 6.5 for the E23A mutant (Fig. 3). The very minor change of the pKa upon substitution of
16 the threonine by lysine, indicates that these residues must be located far from the
17 catalytic site in the ferric state, and thus do not interact with its ligands, even at basic pH
18 (hydroxide bound form). This is in contrast with what was observed for the *D. baarsii*
19 2Fe-SOR, where upon substitution of the lysine of the -EKHVP- motif a substantial shift
20 of the pKa was observed (from 9.0 for the wt to 7.6 for the mutant (Nivière *et al.*, 2004).
21 This change was attributed to the fact that in the basic form the lysine side chain
22 establishes a hydrogen bond with the iron hydroxide ligand (Lys 48 in this enzyme).
23 Upon substitution of the glutamate 23 by an alanine residue a decrease of the apparent
24 pKa of almost 4 units (Table 1). A similar pKa shift was observed in other SORs
25 (Rodrigues *et al.*, 2006, Rodrigues *et al.*, 2007, Nivière *et al.*, 2004) and is thought to

reflect a facilitated binding of a hydroxide anion in the absence of the carboxylate ligand. In fact, resonance Raman spectroscopy demonstrated that upon dissociation of glutamate or H₂O ionization at basic pH an OH⁻ group binds to the iron (Mathé *et al.*, 2005, Todorovic *et al.*, 2009).

The resonance Raman spectra of the ferric wild type, E23A and T24K proteins obtained upon excitation into the (Cys)S → Fe³⁺ charge transfer band display, in the low frequency region, vibrational modes involving contributions from Fe-S(Cys) stretching and internal deformations of the cysteine residue (Fig. 3, Table 1). The spectra of the three proteins are remarkably alike (Table 1), and the observed bands show frequencies and relative intensities comparable to those reported for the SOR from *Desulfoarculus baarsii* (Mathé *et al.*, 2007). The most intense bands, which carry the highest Fe-S(Cys) stretching character, are found at 294-297 cm⁻¹ and 317-320 cm⁻¹ in the spectra of the SORs from *I. hospitalis* and from *D. baarsii*. The 360 cm⁻¹ band, assigned to a Cys deformation mode that contains significant C-N character (Clay, Jenney, Noh, *et al.*, 2002), was also found to be strong in the SORs from *D. baarsii* and *Treponema pallidum*, but not in the spectra of the enzymes from *Archaeoglobus fulgidus* or *Nanoarchaeum equitans* 1Fe-SORs (Mathé *et al.*, 2006, Mathé *et al.*, 2005, Todorovic *et al.*, 2009).

Further insights into the subtle structural changes of the active sites brought up by the point mutations can be derived from the intensity weighted frequency, $\nu_{\text{Fe-S}}$. It is an average frequency calculated from all modes that contain contributions from Fe-S stretching vibrations, weighted by the respective intensities (Blair *et al.*, 1985, Durao *et al.*, 2008). The values of $\nu_{\text{Fe-S}}$ determined here for wild type, E23A, and T24K *I. hospitalis* SORs are 305, 306 and 325 cm⁻¹, respectively, revealing some modifications of the electronic configuration of the active site in T24K (it should be noted that the

component analysis of RR spectra reveals different relative intensities of the component bands, Table 1, leading to distinct intensity weighted frequencies in the mutant enzymes, in spite of their similar experimental RR spectra). The intensity weighted frequency is inversely proportional to the length of the metal-S(Cys) bond, providing insights into its strength. Furthermore, the covalency of the Fe-S bond was proposed to be determinant for the reactivity of SORs (Dey *et al.*, 2007). We have previously observed that the $\nu_{\text{Fe-S}}$ shows a correlation with the kinetics of the release of the OOH-bound peroxide intermediate in SORs from different sources (Todorovic *et al.*, 2009), or iron-superoxo intermediate, as more recently proposed (Bonnot *et al.*, 2012). Based on $\nu_{\text{Fe-S}}$ values, a shorter Fe-S bond and a relatively faster release of the iron-bound superoxo/hydroperoxide intermediate can be predicted in the T24K mutant, in comparison to the wild type and E23A proteins, as it was indeed observed (Table 1). The $\nu_{\text{Fe-S}}$ values fall above (T24K) and below (wild type and E23A) those previously reported for the 1Fe-SORs from *A. fulgidus*, wild type and E12V and E12Q mutants (Todorovic *et al.*, 2009). The Fe-S covalency is sensitive to different influences, e. g. conformational changes of the thiolate ligands (Yachandra *et al.*, 1983), and altered water H-bonding interactions (Sun *et al.*, 2010). The molecular origin of covalency change in T24K is most likely complex in nature, since the immediate Fe-S environment (i. e. geometry, electrostatics and H-bonding network) is altered upon substitution of a basic amino acid by a less bulky, uncharged and polar residue.

The reduction potentials of the SORs were determined to be +235 mV, + 283 mV and +195 mV at pH 7, for the wild type, E23A, and T24K enzymes, respectively (Fig. 4, Table 1), in the range of values reported for the other SORs (+190 to +365 mV)(Emerson *et al.*, 2002, Lombard *et al.*, 2001, Rodrigues *et al.*, 2006, Rodrigues *et*

al., 2007, Testa *et al.*, 2011) and suitable for oxidation/reduction of the superoxide anion (E'_0 of -160 mV and +890 mV for one-electron reduction of O_2 and one-electron reduction of O_2^- , respectively (Koppenol *et al.*, 2010)). The E'_0 values of the wild type and T24K proteins do not change from pH 7 to pH 5 (data not shown), while for the E23A mutant it undergoes a substantial shift to +550 mV, at pH 5. At this pH, the ferric iron of the E23A protein is presumably bound to a H_2O molecule, which is deprotonated at pH 7. This large change of the reduction potential may be explained by the protonation of the hydroxide ligand to form a water-bound molecule that as a neutral species destabilizes the ferric form of the enzyme when compared with its anionic form, thereby increasing its reduction potential. Similar observations were reported for E12Q and E12V mutants of 1Fe-SOR from *A. fulgidus* and E47A mutant of 2Fe-SOR from *D. baarsii* (Abreu *et al.*, 2000, Nivière *et al.*, 2004).

Taken together, the spectroscopic data indicate that the absence of the lysine and glutamate residues did not affect significantly the intrinsic properties of the metal site in *I. hospitalis* SORs.

Kinetics studies- Superoxide dismutation

The superoxide dismutation reaction of the *I. hospitalis* SORs was studied by fast kinetic pulse radiolysis. Initial experiments were carried out by monitoring the consumption of superoxide at 260 nm in the presence and absence of the enzymes (Fig. 5): the consumption of O_2^- occurs through both, the bimolecular disproportionation process (equation 2) and the reaction with the enzyme

The trace for the wild type protein (Fig. 5) can be fitted to a mechanism involving simultaneous first order and second order processes. At room temperature, these

processes are hard to separate but increasing the temperature to 65 °C leads to a better separation of the enzymatic removal of superoxide from the spontaneous dismutation of $O_2^{\cdot-}$. The finding that the rate is clearly measurable at 65 °C suggests that there is a real catalytic rate of superoxide dismutation by the SOR, which is controlled by the reduction of the ferric enzyme by superoxide, since the rate of superoxide reduction by the ferrous form is much faster (see below, and, e.g., refs....). The $O_2^{\cdot-}$ removal by the SOR from *I. hospitalis* at 65 °C and pH 7.1 occurs with a rate constant of $4.8 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ for the wild type enzyme and $5.0 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $4.4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, pH 7.4, for the mutants E23A and T24K, respectively. These values are very similar to the previously reported rate constant for $O_2^{\cdot-}$ removal by the 1Fe-SOR from *A. fulgidus* ($5.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.1 and 83°C (Abreu *et al.*, 2000)), and about three orders of magnitude lower than those of canonical SODs ($\sim 10^9 \text{ M}^{-1}\text{s}^{-1}$). The data for the mutants show that the absence of the sixth ligand of the ferric ion does not lead to an increase of the superoxide oxidation rate, as one could expect if that ligand would somehow hamper the access of the substrate to the iron site, namely if the reaction would occur by an inner sphere mechanism as in canonical SODs (reviewed in, e.g., (Sheng *et al.*, 2014)).

Kinetics studies- Superoxide reduction

The mechanism of superoxide reduction by *I. hospitalis* SORs was investigated by pulse irradiating the reduced enzymes (40-80 μM) with substoichiometric amounts of $O_2^{\cdot-}$ (1.8-2.4 μM). The resultant absorbance changes for the wild type and the two mutant proteins are similar. After pulsing the reduced enzyme with superoxide, an intermediate T1 is formed in a pseudo first-order process, with rates approaching diffusion limit, as observed so far for all SORs (Fig. 6, Table 1). The molecular structure of this intermediate (here labeled as T1) is still controversial. It was proposed that it corresponds to an Fe^{3+} -hydroperoxo species (Silaghi-Dumitrescu *et al.*, 2003), or

alternatively that it originates from a Fe^{2+} -superoxo form that readily decays into a short lived Fe^{3+} -hydroperoxo species (Bonnot *et al.*, 2012)(see Figure 7). This first transient species has a characteristic visible spectrum (Fig. 6) with a maximum at 590 nm and an extinction coefficient of $3800 \text{ M}^{-1}\text{cm}^{-1}$. The formation of T1, within experimental limitations, appears to be pH independent; this intermediate, for the *I.hospitalis* enzymes decays directly into the final oxidized species, the Glu-bound Fe^{3+} form (wild type and T24K mutant), or a presumably $\text{H}_2\text{O}/\text{OH}^-$ -bound form (depending on the pH) for the E23A mutant. This pseudo-first order process is pH-dependent at low pH values and can be interpreted by the following equation: $k_2(\text{obs}) = k_2' [\text{H}^+] + k_2''$, where k_2' is a second order rate constant of protonation and k_2'' a first order rate constant (Emerson *et al.*, 2002). At acidic pH values, the decay of T1 is proportional to the proton concentration and occurs with a diffusion controlled second-order rate constant of $1.50 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ (k_2'); at basic pH values, the decay is pH independent with a first-order rate of 30.3 s^{-1} (k_2'') and likely involves a water molecule (Fig. 6). The pH independent process was also measured for the T24K mutant, being $k_2'' = 64.2 \text{ s}^{-1}$. It was not possible to reach a pH independent region (k_2 at pH 7.0 is 320 s^{-1}) for the E23A mutant due to protein instability at basic pH values, as seen by the decrease of the rate k_2 and the decay of the absorption of the oxidized species (OH-bound Fe^{3+}) upon successive pulses of superoxide anion. The macroscopic mechanism here observed for the *I. hospitalis* enzyme is similar to that of , e.g., the 2-Fe SOR from *D. vulgaris*, in which also a single intermediate was detected, but contrasts to those earlier described for the enzymes from *Archaeoglobus fulgidus*, *Desulfoarculus baarsii* and *Treponema pallidum* (refs). For those proteins a second intermediate is detected, being formed also in a pH dependent form, with electronic features similar to those of the hydroxide-bound ferric state; this second intermediate decays subsequently into the oxidized form through an intramolecular process (Figure 7).

1 Conclusion remarks

2 The SOR from *Ignicoccus hospitalis* does not have the lysine in the typically conserved
3 –EKHVP- motif, representing therefore a natural choice for studying the role of this
4 residue in the mechanism of superoxide reduction. The E23A mutant represents a good
5 model for the putative eukaryotic enzymes, which at the same time lack the highly
6 conserved glutamate ligand of the ferric form. We have shown that the general
7 electronic, kinetic and thermodynamic properties of the *I. hospitalis* SORs are barely
8 affected by the absence of the lysine or the introduced mutations. Accordingly, the
9 studied *I. hospitalis* SORs are indeed *bona fide* superoxide reductases. This finding
10 indicates that the putative SORs from Eukaryotes, which also lack these amino acids,
11 are most probably quite efficient SORs too.

12 The reaction mechanism of *I. hospitalis* SOR involved only one observable
13 intermediate, T1, the ferric-hydroperoxo or ferrous-superoxide species, which decays
14 macroscopically directly to the final oxidized (ferric), resting form. The electronic
15 properties of T1 and the rate constants for its formation and decay are of the same
16 order of magnitude as those reported for the other SORs, *i.e.*, the substitution of lysine
17 has no apparent effect on catalysis. This may signify that i) the lysine is not important
18 for the catalytic mechanism (in agreement with the small effect of its mutation on the
19 SOR from *Desulfovibrio vulgaris* (Emerson *et al.*, 2002)), or ii) that another residue
20 compensates for its absence.

21 The attempt to recover/increase the rate of T1 formation by constructing the T24K
22 mutant, *i.e.* by ‘reverse engineering’ of a conventional SOR, was not successful as no
23 significant differences were observed; instead the rate of the first step of the mechanism
24 was even lower and the rate of T1 release higher. This may suggest that the mutation
25 perturbs the Fe-S (Cys) bond, as indicated by resonance Raman data, without

1 disturbing the positive patch around the active site, or that the substrate accessibility
2 was somehow affected by this mutation.

3 In summary, our data show that a SOR lacking the glutamate and lysine residues close
4 to the active site can be both a competent superoxide reductase and a reasonably
5 effective superoxide dismutase. Clearly, evolution led to development of alternative
6 solutions for equally efficient superoxide detoxification.

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Figure Legends

Fig. 1. Amino acid sequence alignment of SORs, performed with ClustalX (Thompson *et al.*, 1997). Residues that bind the catalytic center are indicated by an asterisk (*); the residues indicated in cardinal (#) correspond to the positions of the highly conserved lysine and glutamate residues; the strictly conserved residues (within this subset) are shaded in black, less conserved ones are highlighted by different grey shades. SORs lacking the lysine residue (ExHxP): *Ignicoccus hospitalis* (gi|156938135), *Hyperthermus butylicus* (gi|124028022), *Thermofilum pendens* (gi|119720733), *Desulfurococcus mucosus* (gi|320101022), *Archaeoglobus profundus* (gi|284161748), *Staphylothermus marinus* (gi|126464958), *Staphylothermus hellenicus* (gi|297526761), *Desulfurococcus kamchatkensis* (gi|218883740), *Thermosphaera aggregans* (gi|296242328); SORs lacking the glutamate residue (xKHxP)- *Guillardia theta* (gi|428172296), *Thalassiosira pseudonana* (gi|224011523), *Thalassiosira oceanica* (gi|397582643), *Nanoarchaeum equitans* (gi|41614807); SORs having both the glutamate and lysine residues (EKHxP): *Holdemania filiformis* (gi|223985741), *Phaeodactylum tricornutum* (gi|219112519), *Giardia lamblia* (gi|308163191), *Archaeoglobus fulgidus* (gi|3914137), *Pyrococcus furiosus* (gi|6066244); SORs lacking both the glutamate and lysine residues (xxHxP): *Beggiatoa sp.* (gi|153875679), *Monosiga brevicollis* (gi|167525365), *Salpingoeca sp.* (gi|326436733). A- Archaea; B- Bacteria; E- Eukarya.

Fig. 2. Top Panel: UV-visible spectra of *I. hospitalis* SOR wild type and the E23A and T24K mutant proteins (note that the enzymes are isolated in variable mixtures of oxidized and reduced forms). A) Spectra at low/neutral pH (100 mM BisTris propane and MES buffer): pH 7 for wild type protein and T24K, pH 5 for E23A; B) Spectra at high pH (100 mM BisTris propane and CAPS buffer): pH 11.5 for wild type protein and T24K, pH 7 for E23A. The spectra are offset vertically. Bottom panel: pH titration curves

of C) wild type, D) E23A and E) T24K proteins, obtained by following the absorbance at 562 nm as function of pH. The solid lines were generated assuming a single-protonation equilibrium with pKa of 10.5 for wild type, 6.5 for E23A and 10.7 for T24K proteins.

Figure 3. Low frequency region of the resonance Raman spectra of *I. hospitalis* SOR wild type: experimental (black trace), component (green trace) and overall component (blue trace) spectra. Inset. Low frequency region of the resonance Raman spectra of E23A and T24K mutants of SOR from *I. hospitalis*. Experimental spectra were recorded at 77 K, with 3 mM ferric protein, 647 nm excitation line, 7 mW laser power and 40 s.

Fig. 4. Redox titrations of wild type (open circles), E23A (solid circles) and T24K (open triangles) SORs from *I. hospitalis*, at pH 7. The titration curves were obtained measuring the absorbance at 636 nm for wild type and T24K and at 580 nm for E23A; the solid lines correspond to Nernst equations with $n=1$ and reduction potentials of 230 mV (wild type), 283 mV (E23A) and 195 mV (T24K).

Fig. 5. Pulse radiolysis assays for the catalytic consumption of superoxide anion ($O_2^{\cdot-}$, 2.6 μ M) followed at 260 nm. The assays were performed in 0.1 M phosphate buffer, pH 7.1 for wild type and pH 7.4 for mutant proteins, in the presence of 50 mM formate and 10 μ M EDTA, in an aerobic atmosphere, at 65° C.

Fig. 6. Formation (A) and decay (B) of the T1 intermediate, measured at 600 nm following the pulse radiolysis of 40 μ M *I. hospitalis* SOR (wild type), with 0.6-3.6 μ M $O_2^{\cdot-}$. A) formation of T1 in a range of less than 0.1 ms, in a pseudo first-order process. B) T1 decay into the oxidized species in a 40 ms process. C) reconstructed visible spectra of the two species formed, T1 (open circles) and final, oxidized, species (solid circles). Molar extinction coefficients were calculated assuming a quantitative 1:1 molar ratio of superoxide with ferrous $[Fe(His)_4(Cys)]$ sites. D) pH dependence of rate constant k_2 for wild type and mutant SORs observed upon the decay of T1 in a range of pH from 5 to 9.

1 The black curves correspond to the fitting of the data to the equation $k_2 (\text{obs}) = k_2' [\text{H}^+] + k_2''$, with $k_2' = 1.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ and $k_2'' = 30.3 \text{ s}^{-1}$ for the wt, $k_2' = 2.25 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ and $k_2'' = 64.2 \text{ s}^{-1}$ for T24K and $k_2' = 1.75 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ for E23A; k_2'' for the E23A mutant could not be determined due to protein instability at basic pH. All solutions were aerobic in 2 mM Tris-HCl, 6.6 mM NaCl, 50 mM of formate at pH 5.5 (except for D).

6 **Fig. 7.** Schematic representation of the catalytic mechanism for the SORs from *I. hospitalis*. The two hypotheses for the only detected intermediate are shown between brackets. Note that for other enzymes the decay from T1 to the oxidized form occurs through a second observable intermediate (T2). Adapted from [review and Bonnot, 2012]

Table 1. Spectroscopic (UV-visible spectra maxima), reduction potentials, frequencies, relative intensities and intensity weighted frequencies ($\langle \nu \rangle_{\text{Fe-S}}$) of the resonance Raman modes with Fe-S(Cys) character, pKas and kinetic properties of wild type, E23A and T24K SORs from *I. hospitalis* at pH 7.0.

	λ_{max} of oxidized form (nm)		E^0 (pH 7.0) (mV)	pKa	ν (cm^{-1})	I^{rel} (a.u.)*	$\langle \nu \rangle_{\text{Fe-S}}$ (cm^{-1})	T1 λ_{max} (nm)	k_1 (T1) ($\times 10^9 \text{ M}^{-1} \text{ s}^{-1}$)	$k_{2,\text{obs}}$ pH7 (s^{-1})
	Acidic form	Basic form								
<i>I. hospitalis</i> wt	640	550	235	10.5	294.7 302.0 310.3 317.6 359.6	1.0 0.26 0.35 0.7 0.8	305	590	0.7	~75
<i>I. hospitalis</i> E23A	660	550	283	10.7	294.3 302.6 312.3 318.0 359.0	1.0 0.21 0.38 0.82 0.64	306	590	1.2	~320
<i>I. hospitalis</i> T24K	650	550	195	6.5	296.6 303.0 311.8 319.9 361.1	1.0 0.3 0.33 0.9 0.82	325	590	0.4	~500

*(a.u.- arbitrary units)

References

- Abreu, I. A., Saraiva, L. M., Carita, J., Huber, H., Stetter, K. O., Cabelli, D. & Teixeira, M. (2000). *Mol Microbiol* **38**, 322-334.
- Abreu, I. A., Saraiva, L. M., Soares, C. M., Teixeira, M. & Cabelli, D. E. (2001). *J Biol Chem* **276**, 38995-39001.
- Blair, D. F., Campbell, G. W., Schoonover, J. R., Chan, S. I., Gray, H. B., Malmstrom, B. G., Pecht, I., Swanson, B. I., Woodruff, W. H., Cho, W. K., English, A. M., Fry, H. A., Lum, V. & Norton, K. A. (1985). *J Am Chem Soc* **107**, 5755-5766.
- Bonnot, F., Molle, T., Menage, S., Moreau, Y., Duval, S., Favaudon, V., Houee-Levin, C. & Niviere, V. (2012). *J Am Chem Soc* **134**, 5120-5130.
- Chen, L., Sharma, P., Le Gall, J., Mariano, A. M., Teixeira, M. & Xavier, A. V. (1994). *Eur J Biochem* **226**, 613-618.
- Clay, M. D., Jenney, F. E., Jr., Hagedoorn, P. L., George, G. N., Adams, M. W. & Johnson, M. K. (2002). *J Am Chem Soc* **124**, 788-805.
- Clay, M. D., Jenney, F. E., Jr., Noh, H. J., Hagedoorn, P. L., Adams, M. W. & Johnson, M. K. (2002). *Biochemistry* **41**, 9833-9841.
- Dey, A., Jenney, F. E., Adams, M. W. W., Johnson, M. K., Hodgson, K. O., Hedman, B. & Solomon, E. I. (2007). *J Am Chem Soc* **129**, 12418-12431.
- Durao, P., Chen, Z., Silva, C. S., Soares, C. M., Pereira, M. M., Todorovic, S., Hildebrandt, P., Bento, I., Lindley, P. F. & Martins, L. O. (2008). *Biochem J* **412**, 339-346.
- Emerson, J. P., Coulter, E. D., Cabelli, D. E., Phillips, R. S. & Kurtz, D. M., Jr. (2002). *Biochemistry* **41**, 4348-4357.
- Fischer, D. S., and Price, D. C. (1964). *Clin Chem* **10**, 21-31.
- Goto, J. J., Gralla, E. B., Valentine, J. S. & Cabelli, D. E. (1998). *J Biol Chem* **273**, 30104-30109.
- Katona, G., Carpentier, P., Niviere, V., Amara, P., Adam, V., Ohana, J., Tsanov, N. & Bourgeois, D. (2007). *Science* **316**, 449-453.
- Koppenol, W. H., Stanbury, D. M. & Bounds, P. L. (2010). *Free Radic Biol Med* **49**, 317-322.
- Kratzer, C., Welte, C., Dorner, K., Friedrich, T. & Deppenmeier, U. (2011). *FEBS J* **278**, 442-451.
- Kurtz, D. M., Jr. (2006). *J Inorg Biochem* **100**, 679-693.
- Laemmli, U. K. (1970). *Nature* **227**, 680-685.
- Lombard, M., Houee-Levin, C., Touati, D., Fontecave, M. & Niviere, V. (2001). *Biochemistry* **40**, 5032-5040.
- Lombard, M., Touati, D., Fontecave, M. & Niviere, V. (2000). *J Biol Chem* **275**, 27021-27026.
- Lucchetti-Miganeh, C., Goudenege, D., Thybert, D., Salbert, G. & Barloy-Hubler, F. (2011). *BMC Microbiol* **11**, 105.
- Mathé, C., Nivière, V., Houee-Levin, C. & Mattioli, T. A. (2006). *Biophys Chem* **119**, 38-48.
- Mathé, C., Nivière, V. & Mattioli, T. A. (2005). *J Am Chem Soc* **127**, 16436-16441.
- Mathé, C., Weill, C. O., Mattioli, T. A., Berthomieu, C., Houee-Levin, C., Tremey, E. & Nivière, V. (2007). *J Biol Chem* **282**, 22207-22216.
- Moura, I., Tavares, P., Moura, J. J., Ravi, N., Huynh, B. H., Liu, M. Y. & LeGall, J. (1990). *J Biol Chem* **265**, 21596-21602.

- 1 Nivière, V., Asso, M., Weill, C. O., Lombard, M., Guigliarelli, B., Favaudon, V. & Houee-Levin, C.
2 (2004). *Biochemistry* **43**, 808-818.
- 3 Nivière, V. & Lombard, M. (2002). *Methods Enzymol* **349**, 123-129.
- 4 Nivière, V., Lombard, M., Fontecave, M. & Houee-Levin, C. (2001). *FEBS Lett* **497**, 171-173.
- 5 Pinho, F. G., Romao, C. V., Pinto, A. F., Saraiva, L. M., Huber, H., Matias, P. M., Teixeira, M. &
6 Bandejas, T. M. (2010). *Acta Crystallogr Sect F Struct Biol Cryst Commun* **66**, 605-607.
- 7 Pinto, A. F., Rodrigues, J. V. & Teixeira, M. (2010). *Biochim Biophys Acta* **1804**, 285-297.
- 8 Podar, M., Anderson, I., Makarova, K. S., Elkins, J. G., Ivanova, N., Wall, M. A., Lykidis, A.,
9 Mavromatis, K., Sun, H., Hudson, M. E., Chen, W., Deciu, C., Hutchison, D., Eads, J. R.,
10 Anderson, A., Fernandes, F., Szeto, E., Lapidus, A., Kyrpides, N. C., Saier, M. H., Jr.,
11 Richardson, P. M., Rachel, R., Huber, H., Eisen, J. A., Koonin, E. V., Keller, M. & Stetter, K.
12 O. (2008). *Genome Biol* **9**, R158.
- 13 Rodrigues, J. V., Abreu, I. A., Cabelli, D. & Teixeira, M. (2006). *Biochemistry* **45**, 9266-9278.
- 14 Rodrigues, J. V., Saraiva, L. M., Abreu, I. A., Teixeira, M. & Cabelli, D. E. (2007). *J Biol Inorg Chem*
15 **12**, 248-256.
- 16 Rodrigues, J. V., Victor, B. L., Huber, H., Saraiva, L. M., Soares, C. M., Cabelli, D. E. & Teixeira, M.
17 (2008). *J Biol Inorg Chem* **13**, 219-228.
- 18 Sheng, Y., Abreu, I. A., Cabelli, D. E., Maroney, M. J., Miller, A. F., Teixeira, M. & Valentine, J. S.
19 (2014). *Chem Rev* **114**, 3854-3918.
- 20 Silaghi-Dumitrescu, R., Silaghi-Dumitrescu, I., Coulter, E. D. & Kurtz, D. M., Jr. (2003). *Inorg*
21 *Chem* **42**, 446-456.
- 22 Sit, P. H., Zipoli, F., Chen, J., Car, R., Cohen, M. H. & Selloni, A. (2011). *Chemistry* **17**, 12136-
23 12143.
- 24 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D.,
25 Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985). *Anal Biochem* **150**, 76-85.
- 26 Sun, N., Dey, A., Xiao, Z., Wedd, A. G., Hodgson, K. O., Hedman, B. & Solomon, E. I. (2010). *J Am*
27 *Chem Soc* **132**, 12639-12647.
- 28 Testa, F., Mastronicola, D., Cabelli, D. E., Bordin, E., Pucillo, L. P., Sarti, P., Saraiva, L. M., Giuffrè,
29 A. & Teixeira, M. (2011). *Free Radic Biol Med* **51**, 1567-1574.
- 30 Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). *Nucleic Acids*
31 *Res* **25**, 4876-4882.
- 32 Todorovic, S., Rodrigues, J. V., Pinto, A. F., Thomsen, C., Hildebrandt, P., Teixeira, M. & Murgida,
33 D. H. (2009). *Phys Chem Chem Phys* **11**, 1809-1815.
- 34 Yachandra, V. K., Hare, J., Moura, I. & Spiro, T. G. (1983). *J Am Chem Soc* **105**, 6455-6462.
- 35 Yeh, A. P., Hu, Y., Jenney, F. E., Jr., Adams, M. W. & Rees, D. C. (2000). *Biochemistry* **39**, 2499-
36 2508.