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Role of Protons in Superoxide Reduction by a Superoxide Reductase Analogue

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Superoxide reduction by thiolate-ligated $[Fe^{II}(S^{Me2}N_4(tren))]^+$ (**1**) involves two proton-dependent steps and a single peroxide intermediate, $[Fe^{III}(S^{Me2}N_4(tren))(OOH)]^+$ (**2**). An external proton donor is required, ruling out mechanisms involving H^+ or H-atom abstraction from the ligand N−H. The initial protonation step affording **2** occurs with fairly basic proton donors (EtOH, MeOH, NH_4^+) in THF. More acidic proton donors are required to cleave the Fe−O(peroxide) bond in MeOH, and this occurs via a dissociative mechanism. Reaction rates are dependent on the pK_a of the proton donor, and a common $[Fe^{III}(S^{Me2}N_4(tren))(MeOH)]^{2+}$ (**3**) intermediate is involved. Acetic acid releases H_2O_2 from **2** under pseudo-first-order conditions ([HOAc] = 138 mM, [**2**] = 0.49 mM) with a rate constant of 8.2×10^{-4} s^{-1} at −78 °C in MeOH. Reduction of **3** with Cp_2Co regenerates the active catalyst **1**.

Superoxide reductases (SORs) are non-heme iron enzymes that reduce superoxide (O_2^-) to H_2O_2 in anaerobic microbes.[1a−j] The catalytically active form of this enzyme contains reduced Fe^{2+} ligated by four N^{his} and a S^{cys} trans to an open coordination site (Figure 1).[1h,i] The oxidized SOR resting state contains a highly conserved Glu-CO_2^- (^{14}Glu or ^{47}Glu) coordinated to this site.[1c,e,h] Upon reduction, this Glu-CO_2^- dissociates to regenerate the open binding site necessary for catalysis (reaction 3, Figure 1). The mechanism by which

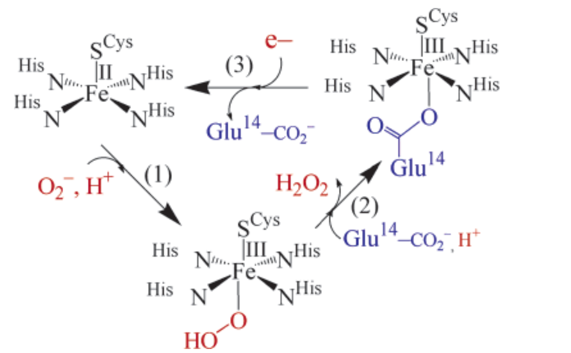

Figure 1. Proposed mechanism of superoxide reduction by the non-heme iron enzyme superoxide reductase (SOR).

SOR is proposed to reduce superoxide has been controversial, particularly with regard to the number of intermediates involved.[1a,b,e,g,2] It is generally agreed that the mechanism involves initial coordination of O_2^- to the open coordination site and is thus inner-sphere.[1f] After this step, two intermediates are observed in the reaction between *D. baarsii* SOR and O_2^-,[1b,2] whereas only one intermediate is observed in the reaction between *D. vulgaris* SOR and O_2^-.[1a,e,g] DFT calculations indicate that an end-on Fe^{III}−OOH intermediate is most likely involved.[1g] It has been suggested that the nearby ^{47}Glu-CO_2^- displaces peroxide from the final intermediate.[1b,c] However, it is not clear whether the Glu-bound state is involved in the catalytic cycle or whether a solvent-bound form[1a] would be catalytically more competent. Protons clearly play an important role in the SOR mechanism. The source of protons (in both the first and second protonation steps) has also been a point of discussion. Under acidic conditions, ^{14}Glu-COOH has been proposed to serve as a proton source.[1a,e] Solvent was shown to provide protons in the *D. baarsii* SOR mechanism.[1b] A highly conserved Lys-NH_3^+ residue (^{15}Lys or ^{48}Lys), essential for catalytic activity,[2] has been shown to affect rates of H_2O_2 formation, thus suggesting its involvement in the mechanism.[1a,2] A condensed version of the SOR mechanism is presented in Figure 1.

The active site of SOR closely resembles that of P450,[3] and both enzymatic mechanisms are proposed to involve an

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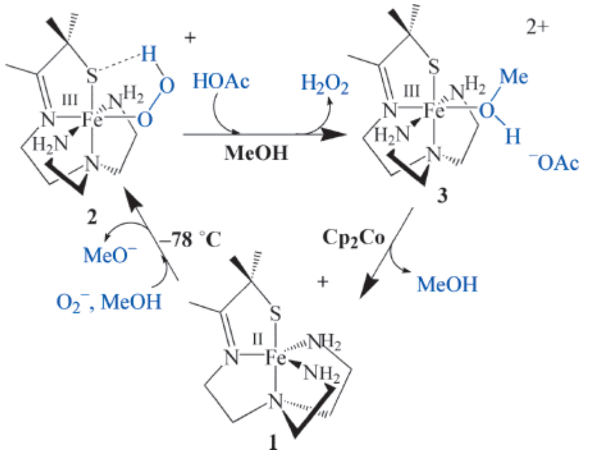

Figure 2. Biomimetic SOR analogue reduces superoxide in two proton-dependent steps via an Fe^{III}−OOH intermediate **2**. The reduced catalyst **1** is regenerated via HOAc-promoted H_2O_2 release, followed by reduction with cobaltocene.

Fe^{III}−OOH intermediate and initial protonation of the distal oxygen. The site to which the second proton is delivered, as well as the spin state,[4b] determines whether the Fe−O or O−O bond is cleaved.[4a−c] Double protonation of the distal oxygen and an $S = 1/2$ spin state[4b] would result in heterolytic O−O bond cleavage to form H_2O and a high-valent Fe^{V}=O species, whereas protonation of the proximal oxygen and an $S = 5/2$ spin state, would result in Fe−O bond cleavage to afford H_2O_2. The only observed peroxide in SOR is $S = 5/2$ (generated via H_2O_2 addition to a Glu → Ala mutant),[1c] thus it is likely that H_2O_2 release by SOR involves protonation at the Fe^{III}−OOH proximal oxygen.

Our group has demonstrated that thiolate-ligated $[Fe^{II}(S^{Me2}N_4(tren))]^+$ (**1**) (Figure 2) will react with O_2^- in MeOH at low temperatures to generate the first synthetic example of an Fe^{3+}-OOH species containing sulfur in the coordination sphere, $[Fe^{III}(S^{Me2}N_4(tren))(OOH)]^+$ (**2**).[5a,b] Intermediate **2** is low-spin ($S = 1/2$) and displays a ν_{O-O} at 784 cm^{-1} (that shifts to 753 cm^{-1} upon isotopic labeling with $^{18}O_2^-$), a charge-transfer transition at 455 (2900) nm, and a coordinated diatomic oxygen ligand with one short, and one long Fe−O distance at 1.86(3) and 2.78(3) Å, respectively, as determined by EXAFS.[5a] The charge-transfer transition associated with **2** is unusually high in energy for a S → Fe(III) CT transition.[6,7]

In this work, we explore the addition of proton and electron sources to our functional model[5a] in order to model reaction steps 1−3 of the proposed SOR mechanism (Figure 1). Preisolation and purification of the extremely air sensitive Fe^{II} precursor, **1**, has afforded a much cleaner reaction system and a significantly more stable Fe^{III}−OOH (**2**) species, thus allowing us to probe its reaction chemistry.[8] We can separately monitor the first and second protonation steps involved in H_2O_2 formation by our model, but this requires the use of two different solvents. The first protonation step is most effectively monitored in THF, whereas the second protonation step is cleanest when monitored in MeOH. In rigorously dried THF, no reaction is observed between prepurified **1** and O_2^- (solubilized as the 18-cr-6-K^+ salt) until an external proton source is added (Figure 2).[8] This rules out a mechanism involving H^+ or H-atom abstraction[9] from the ligand N−H's. Addition of a proton source such as NH_4^+, MeOH, or EtOH to a mixture containing prepurified **1** + 1 equiv of O_2^- in THF at −78 °C rapidly affords our metastable hydroperoxide species $[Fe^{III}(S^{Me2}N_4(tren))(OOH)]^+$ (**2**). Rates of **2** formation are dependent on the pK_a of the proton donor: for the reaction to occur at comparable rates, the concentration of EtOH has to be ~500 times higher than that of NH_4^+. Although the pK_a's (both relative and absolute) of these proton donors are likely to differ dramatically in THF (vs H_2O), the fact that EtOH will protonate **2** suggests that the initial protonation site is rather basic.[10,11] The proton dependence of **2** formation, along with the unusually high energy of the S → Fe(III) charge-transfer band[6] and the highly ordered distal oxygen observed by EXAFS,[5a] suggests that the distal peroxide oxygen is protonated and perhaps hydrogen-bonded to the thiolate. A hydrogen-bonded ring structure (Figure 2) might, in fact, provide a driving force for the formation of hydroperoxide-ligated **2**.

The second protonation step in the reduction of superoxide by our model requires stronger acids, such as HOAc, HBF_4, or $HClO_4$. Weaker acids [NH_4^+ (a Lys analogue) and MeOH] do not release H_2O_2 from **2** in MeOH. Reactions involving acids with noncoordinating anions (HBF_4 or $HClO_4$) cleanly afford a common eggplant purple intermediate (**3**, λ_{max} = 565 nm, Figures S-2 and S-4) in MeOH at −78 °C. This purple intermediate **3** is also observed when HBF_4 is added to $[Fe^{III}(S^{Me2}N_4(tren))(OMe)]^+$ (**6**)[5a] at −78 °C (Figure S-3), suggesting that **3** is the protonated, dicationic, methanol-bound species $[Fe^{III}(S^{Me2}N_4(tren))(MeOH)]^{2+}$ (Figure 2). Acetic acid reacts with peroxide-bound **2** (Figure 3) also to afford **3**, which then converts to acetate-bound $[Fe^{III}(S^{Me2}N_4(tren))(OAc)]^+$ (**4**) upon warming.[12] It is likely that glutamic acid-promoted H_2O_2 release by SOR (reaction 2, Figure 1) occurs via a similar mechanism involving a solvent-bound intermediate. Under pseudo-first-order conditions ([HOAc] = 138 mM, [Fe−OOH] = 0.49 mM), our peroxide species

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(8) When generated from prepurified **1**, **2** is stable for at least 6 h at −78 °C in THF. Previously (ref 5a), it was reported that intractable solids formed when O_2^- was added to **1** in aprotic solvents. Extreme sensitivity to dioxygen (≥2 ppm) and trace amounts water make purification of **1** nontrivial.

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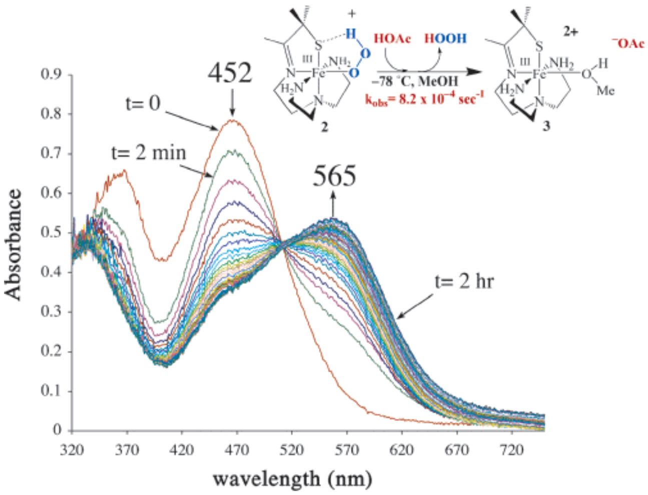

Figure 3. Reaction between **2** and HOAc in MeOH under pseudo-first-order conditions ([HOAc] = 139 mM, [**2**] = 0.49 mM) monitored via electronic absorption spectroscopy at −78 °C (scans taken every 2 min).

2 reacts with HOAc with a rate constant of 8.2×10^{-4} s^{-1} at −78 °C in MeOH. Reaction rates are dependent on the pK_a of the proton donor; reactions are complete in seconds with stronger acids (HBF_4, $HClO_4$) vs hours with HOAc. The fact that a common MeOH-bound intermediate **3** is observed in these reactions suggests that H_2O_2 release occurs via a proton-induced dissociative mechanism. An associative mechanism involving nucleophilic displacement is ruled out by the fact that $NH_4^+OAc^-$ does not release H_2O_2 from **2** in MeOH.[13] That a similar mechanism is involved in the second protonation step of SOR is supported by the fact that the initial product observed during the decay of the SOR peroxide (600 nm) intermediate (under basic conditions) appears to lack a coordinated glutamate and is suggested to be five-coordinate or solvent-bound.[1a]

To mimic the last step in the SOR mechanism (reaction 3 of Figure 1), Cp_2Co was added as an electron source to the oxidized Fe^{III} intermediate **3**, which forms upon HOAc-induced H_2O_2 release from **2**. This resulted in the regeneration of reduced $[Fe^{II}(S^{Me2}N_4(tren))]^+$ (**1**), which subsequently reacts with superoxide to afford the peroxide intermediate **2**. Thus far, we have achieved eight turnovers under these conditions, and we are working on optimizing this catalytic reaction. The key to achieving higher turnovers will require the removal of the H_2O_2 released in the reaction so as to avoid peroxide-induced decomposition of the iron complex.

In conclusion, the work reported herein demonstrates that superoxide reduction by our thiolate-ligated model complex involves two proton-dependent steps involving a single Fe^{III}−OOH intermediate. The site initially protonated appears to be fairly basic. Stronger acids are required to cleave the Fe−O(peroxide) bond and release H_2O_2, which occurs via a dissociative mechanism.[13] A similar mechanism is most likely involved in H_2O_2 release from the SOR enzyme.[1a] The proton dependence of the first step of our mechanism agrees with Sawyer's reported thermodynamic and kinetic data for superoxide-induced oxidation reactions[10] but differs from that of SOR.[1a,b] The cis, instead of trans, arrangement between the thiolate and the peroxide of our model, versus the enzyme, might be responsible for the mechanistic differences between our model and the enzyme. The fact that we observe only one (hydro)peroxide-bound intermediate, as opposed to two, suggests that the mechanism differs from that of the *D. baarsii* SOR enzyme.[1b,2] Possible mechanisms consistent with the proton dependence of the formation of **2** would involve either initial protonation of superoxide to afford HO_2, a more potent oxidant than O_2^-, or initial protonation of the thiolate sulfur of **1** to afford a dicationic species that has a higher affinity for O_2^-. More detailed kinetic studies of both the proton-induced formation of Fe^{III}−OOH (**2**) and the proton-induced release of H_2O_2 are currently underway in our laboratory.

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Supporting Information Available: Experimental section describing compound preparation; kinetic plot for **2** + HOAc (Figure S-1); electronic absorption spectra showing $HClO_4$-induced conversion of **2** to **3** (Figure S-2), HBF_4-induced conversion of **6** to **3** (Figure S-3), and HBF_4-induced conversion of **2** to **3** (Figure S-4). This information is available free of charge via the Internet at http://pubs.acs.org.

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(12) The final products in this reaction were identified via comparison of their electronic absorption spectra with that of authentic samples (synthesized from $[Fe^{III}(S^{Me2}N_4(tren))(MeCN)]^{2+}$ and $Bu_4N^+OAc^-$): Shearer, J.; Fitch, S. B.; Kaminsky, W.; Benedict, J.; Scarrow, R. C.; Kovacs, J. A. *Proc. Natl. Acad. Sci., U.S.A.* **2003**, *100*, 3671−3676.

(13) In the absence of additional kinetic data, we cannot rule out an associative mechanism involving OAc^- binding to the solvent-ligated **3** intermediate.