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Role of Protons in Superoxide Reduction by a Superoxide Reductase Analogue

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Abstract

Superoxide reduction by thiolate-ligated $[\text{Fe}^{\text{II}}(\text{S}^{\text{Me}_2\text{N}_4(\text{tren}))}]^+$ (**1**) involves two proton-dependent steps and a single peroxide intermediate, $[\text{Fe}^{\text{III}}(\text{S}^{\text{Me}_2\text{N}_4(\text{tren}))}(\text{OOH})]^+$ (**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 $\text{p}K_{\text{a}}$ of the proton donor, and a common $[\text{Fe}^{\text{III}}(\text{S}^{\text{Me}_2\text{N}_4(\text{tren}))}(\text{MeOH})]^{2+}$ (**3**) intermediate is involved. Acetic acid releases H_2O_2 from **2** under pseudo-first-order conditions ($[\text{HOAc}] = 138 \text{ mM}$, $[\textbf{2}] = 0.49 \text{ mM}$) with a rate constant of $8.2 \times 10^{-4} \text{ s}^{-1}$ 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 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 $\text{Fe}^{\text{III}}\text{--OOH}$ intermediate is most likely involved.^{1g} It has been suggested that the nearby $^{47}\text{Glu}\text{--CO}_2^-$ 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

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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>.

discussion. Under acidic conditions, ¹⁴Glu-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-NH₃⁺ residue (¹⁵Lys or ⁴⁸Lys), essential for catalytic activity,² has been shown to affect rates of H₂O₂ 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 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 H₂O 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 H₂O₂. The only observed peroxide in SOR is *S* = 5/2 (generated via H₂O₂ addition to a Glu → Ala mutant),^{1c} thus it is likely that H₂O₂ release by SOR involves protonation at the Fe^{III}-OOH proximal oxygen.

Our group has demonstrated that thiolate-ligated [Fe^{II}(S^{Me}₂N₄(tren))]⁺ (**1**) (Figure 2) will react with O₂⁻ in MeOH at low temperatures to generate the first synthetic example of an Fe³⁺-OOH species containing sulfur in the coordination sphere, [Fe^{III}(S^{Me}₂N₄(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 ¹⁸O₂⁻), 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). Pre-isolation 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 H₂O₂ 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 O₂⁻ (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 abstraction⁹ from the ligand N-H's. Addition of a proton source such as NH₄⁺, MeOH, or EtOH to a mixture containing prepurified **1** + 1 equiv of O₂⁻ in THF at -78 °C rapidly affords our metastable hydroperoxide species [Fe^{III}(S^{Me}₂N₄(tren))(OOH)]⁺ (**2**). Rates of **2** formation are dependent on the p*K*_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 NH₄⁺. Although the p*K*_a's (both relative and absolute) of these proton donors are likely to differ dramatically in THF (vs H₂O), 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 $\rightarrow$ Fe(III) charge-transfer band⁶ 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, HBF₄, or HClO₄. Weaker acids [NH₄⁺ (a Lys analogue) and MeOH] do not release H₂O₂ from **2** in MeOH. Reactions involving acids with noncoordinating anions (HBF₄ or HClO₄) 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 HBF₄ is added to [Fe^{III}(S^{Me}₂N₄(tren))(OMe)]⁺ (**6**)^{5a} at -78 °C (Figure S-3), suggesting that **3** is the protonated, dicationic, methanol-bound species [Fe^{III}(S^{Me}₂N₄(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^{Me}₂N₄(tren))(OAc)]⁺ (**4**) upon warming.¹² It is likely that glutamic acid-promoted H₂O₂ 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 **2** reacts with HOAc with a rate constant of $8.2 \times 10^{-4} \text{ s}^{-1}$ 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 (HBF₄, HClO₄) vs hours with HOAc. The fact that a common MeOH-bound intermediate **3** is observed in these reactions suggests that H₂O₂ release occurs via a proton-induced dissociative mechanism. An associative mechanism involving nucleophilic displacement is ruled out by the fact that NH₄⁺ OAc⁻ does not release H₂O₂ 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), Cp₂Co was added as an electron source to the oxidized Fe^{III} intermediate **3**, which forms upon HOAc-induced H₂O₂ release from **2**. This resulted in the regeneration of reduced [Fe^{II}(S^{Me}₂N₄(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 H₂O₂ 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 H₂O₂, which occurs via a dissociative mechanism.¹³ A similar mechanism is most likely involved in H₂O₂ 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 reactions¹⁰ 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 HO₂, a more potent oxidant than O₂^{•-}, or initial protonation of the thiolate sulfur of **1** to afford a dicationic species that has a higher affinity for O₂^{•-}. More detailed kinetic studies of both the proton-induced formation of Fe^{III}-OOH (**2**) and the proton-induced release of H₂O₂ are currently underway in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. (a) Emerson JP, Coulter ED, Cabelli DE, Phillips RS, Kurtz DM Jr. *Biochemistry*. 2002; 41:4348–4357. [PubMed: 11914081] (b) Niviere V, Asso M, Weill CO, Lombard M, Guigliarelli B, Favaudon V, Houe'e-Levin C. *Biochemistry*. 2004; 43:808–818. [PubMed: 14730986] (c) Mathe C, Mattioli TA, Horner O, Lombard M, Latour J-M, Fontecave M, Niviere V. *J Am Chem Soc*. 2002; 124:4966–4967. [PubMed: 11982354] (d) Jenney FE Jr, Verhagen MFJM, Cui X, Adams MWW. *Science*. 1999; 286:306–309. [PubMed: 10514376] (e) Coulter ED, Emerson JP, Kurtz DM Jr, Cabelli DE. *J Am Chem Soc*. 2000; 122:11555–11556. (f) Clay MD, Jenney FE Jr, Hagedoorn PL, George GN, Adams MWW, Johnson MK. *J Am Chem Soc*. 2002; 124:788–805. [PubMed: 11817955] (g) Silaghi-Dumitrescu R, Silaghi-Dumitrescu I, Coulter ED, Kurtz DM Jr. *Inorg Chem*. 2003; 42:446–456. [PubMed: 12693226] (h) Yeh AP, Hu Y, Jenney FE Jr, Adams MWW, Rees DC. *Biochemistry*. 2000; 39:2499–2508. [PubMed: 10704199] (i) Coelho AV, Matias P, Fulop V, Thompson A, Gonzalez A, Carrondo MA. *J Biol Inorg Chem*. 1997; 2:680–689. (j) Jovanovic T, Ascenso C, Hazlett KRO, Sikkink R, Krebs C, Litwiller R, Benson LM, Moura I, Moura JIG, Radolf JD, Huynh B Hanh, Naylor S, Rusnak F. *J Biol Chem*. 2000; 275:28439–28448. [PubMed: 10874033]
2. Lombard M, Houee-Levin C, Touati D, Fontecave M, Niviere V. *Biochemistry*. 2001; 40:5032–5040. [PubMed: 11305919]
3. (a) Sono M, Roach MP, Coulter ED, Dawson JH. *Chem Rev*. 1996; 96:2841–2887. [PubMed: 11848843] (b) Makris TM, Davydov R, Denisov IG, Hoffman BM, Sligar SG. *Drug Metabolism Reviews*. 2002; 34:691–708. [PubMed: 12487147]
4. (a) Harris DL, Loew GH. *J Am Chem Soc*. 1998; 120:8941–8948. (b) Lehnert N, Neese F, Ho RY, Que L Jr, Solomon EI. *J Am Chem Soc*. 2002; 124:10810–10822. [PubMed: 12207537] (c) Neese F, Zaleski JM, Zaleski KL, Solomon EI. *J Am Chem Soc*. 2000; 122:11703–11724.
5. (a) Shearer J, Scarrow RC, Kovacs JA. *J Am Chem Soc*. 2002; 124:11709–11717. [PubMed: 12296737] (b) Shearer J, Nehring J, Kaminsky W, Kovacs JA. *Inorg Chem*. 2001; 40:5483–5484. [PubMed: 11599942]
6. Dey A, Chow M, Theisen RM, Kovacs JA, Solomon EI. manuscript in preparation.
7. Kovacs JA. *Chem Rev*. 2004; 104:825–848. [PubMed: 14871143]
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 O₂^{•-} was added to **1** in aprotic solvents. Extreme sensitivity to dioxygen (> 2 ppm) and trace amounts water make purification of **1** nontrivial.
9. Bernhard P, Anson FC. *Inorg Chem*. 1988; 27:4574–4577.

10. (a) Chin D-H, Chiericato G Jr, Nanni EJ Jr, Sawyer DT. J Am Chem Soc. 1982; 104:1296–1299.
(b) Sawyer DT, Gibian MJ, Morrison MM, Seo ET. J Am Chem Soc. 1978; 100:627–628.
11. The pK_a of H_2O_2 is 11.2 in water 15.8 in MeOH at 25 °C: Buncel E, Chuaqui C, Wilson H. Int J Chem Kinet. 1982; 14:823–837.
12. The final products in this reaction were identified via comparison of their electronic absorption spectra with that of authentic samples (synthesized from $[Fe^{III}(S^{Me}_2N_4(tren))(MeCN)]^{2+}$ and $Bu_4N^+OAc^-$): Shearer J, Fitch SB, Kaminsky W, Benedict J, Scarrow RC, Kovacs JA. Proc Natl Acad Sci USA. 2003; 100:3671–3676. [PubMed: 12655068]
13. In the absence of additional kinetic data, we cannot rule out an associative mechanism involving OAc^- binding to the solvent-ligated **3** intermediate.

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

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.

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