Role of Protons in Superoxide Reduction by a Superoxide Reductase Analogue

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Received August 25, 2004

Superoxide reduction by thiolate-ligated [Fe[II](SMe2N4(tren))]+ (1) involves two proton-dependent steps and a single peroxide intermediate, [Fe[II](SMe2N4(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, NH4+) 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 pKa of the proton donor, and a common [Fe[II](SMe2N4(tren))(MeOH)]2+ (3) intermediate is involved. Acetic acid releases H2O2 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 Cp2Co regenerates the active catalyst 1.

Superoxide reductases (SORs) are non-heme iron enzymes that reduce superoxide (O2−) to H2O2 in anaerobic microbes.1a−3 The catalytically active form of this enzyme contains reduced Fe2+ ligated by four Nδ+ and a Sδ+ trans to an open coordination site (Figure 1).1h,i The oxidized SOR resting state contains a highly conserved Glu-COO2− (14Glu or 47Glu) coordinated to this site.1e,h Upon reduction, this Glu-COO2− dissociates to regenerate the open binding site necessary for catalysis (reaction 3, Figure 1). The mechanism by which

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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 O2− 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 O2−.1a,b,e,g,2 Whereas only one intermediate is observed in the reaction between D. vulgaris SOR and O2−.1a,c DFT calculations indicate that an end-on Fe3+-OOH intermediate is most likely involved.1g It has been suggested that the nearby 47Glu-CO2− displaces peroxide from the final intermediate.1bc However, it is not clear whether the Glu-bound state is involved in the catalytic cycle or whether a solvent-bound form1a 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,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-NHδ+ 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,c 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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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, determines whether the Fe−O or O−O bond is cleaved. Double protonation of the distal oxygen and an S = 1/2 spin state would result in heterolytic O−O bond cleavage to form H₂O₂ and a high-valent Fe⁷⁺=O species, whereas protonation of the proximal oxygen and an S = 3/2 spin state would result in Fe−O bond cleavage to afford H₂O₂. The only observed peroxide in SOR is S = 3/2 (generated via H₂O₂ addition to a Glu−Ala mutant), 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, (SMe₂N₄(tren))²⁺)] (1) (Figure 2) will react with O₂⁻ in MeOH at low temperatures to generate the first synthetic example of an Fe⁺⁺−OOH species containing sulfur in the coordination sphere, [Fe(III)(SMe₂N₄(tren))(OOH)]⁺ (2). Intermediate 2 is low-spin (S = 1/2) and displays a vOOH 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 charge-transfer transition. It is stable for at least 6 h at −78 °C in THF, whereas the second protonation step is clearest 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). This rules out a mechanism involving H⁺ or H-atom abstraction 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)(SMe₂N₄(tren))(OOH)]⁺ (2). Rates of 2 formation are dependent on the pKa 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 pKa’s (both relative and absolute) of these proton donors are likely to differ dramatically in THF (vs H₂O₂), the fact that EtOH will protonate 2 suggests that the initial protonation site is rather basic. The proton dependence of 2 formation, along with the unusually high energy of the S → Fe(III) charge-transfer band and the highly ordered distal oxygen observed by EXAFS 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, λmax = 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)(SMe₂N₄(tren))(OMe)]⁺ (5) at −78 °C (Figure S-3), suggesting that 3 is the protonated, dicatonic, methanol-bound species [Fe(III)(SMe₂N₄(tren))(OMe)]⁺ (Figure 2). Acetic acid reacts with peroxide-bound 2 (Figure 3) also to afford 3, which then converts to acetate-bound [Fe(III)(SMe₂N₄(tren))(OAc)]⁺ (4) upon warming. It is likely that glutamic acid-promoted H₂O₂ release by SOR (reaction 2, Figure 3) 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 is not released.

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 oxygen (≥2 ppm) and trace amounts water make purification of 1 nontrivial.

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2 reacts with HOAc with a rate constant of $8.2 \times 10^{-4}$ 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$_2$O$_2$ 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$_2$O$_2$ from 2 in MeOH. In the absence of additional kinetic data, we cannot rule out an associative mechanism involving OAc$^-$ binding to the solvent-ligated 3 intermediate.

To mimic the last step in the SOR mechanism (reaction 3 of Figure 1), Cp$_2$Co was added as an electron source to the oxidized Fe$^{III}$ intermediate 3, which forms upon HOAc-induced H$_2$O$_2$ release from 2. This resulted in the regeneration of reduced [Fe$^{III}$(SMe$_2$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$_2$O$_2$ 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$_2$O$_2$, which occurs via a dissociative mechanism. A similar mechanism is most likely involved in H$_2$O$_2$ release from the SOR enzyme. The proton dependence of the first step of our mechanism agrees with Sawyer’s reported thermodynamic and kinetic data for superoxide-induced oxidation reactions but differs from that of SOR. 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. 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 dicaticionic 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$_2$O$_2$ are currently underway in our laboratory.

Acknowledgment. This work was supported by NIH Grant GM45881. We thank Priscilla Lugo-Mas for experimental assistance and Leon Xu for helpful discussion.