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A Functional Model for the Cysteinate–Ligated Non–Heme Iron Enzyme Superoxide Reductase (SOR)

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Superoxide reductases (SORs) are cysteinate-ligated non-hemeiron enzymes¹ that reduce superoxide (O_2^-) to H_2O_2 in anaerobic microbes.² The cysteinate of SOR is *trans* to the O_2^- binding site, and is proposed to play an important role in promoting the catalytic reaction. Herein, we report a rare example of a functional metalloenzyme active site model, that reduces O_2^- via *atrans* thiolate–ligated Fe(III)–peroxo intermediate. The *trans* thiolate is shown to lower the redox potential, change the spin-state, and dramatically weaken the Fe–O bond, favoring O_2^- reduction and H_2O_2 release.

Superoxide is a toxic byproduct of dioxygen chemistry that has been linked to a number of disease states.³ The proposed SOR mechanism involves the oxidative addition of O_2^- to the open site of the square pyramidal $Fe^{II}N_4^{His}S^{Cys}$ active site^{2c} to afford a *trans* S^{Cys} –ligated Fe^{III} –peroxo intermediate.^{2d,e} This intermediate displays an intense S-to-Fe(III) charge transfer band at ~600(–3500) nm, but has yet to be characterized by vibrational spectroscopy. Iron-peroxo species are extremely difficult to characterize since they are thermally unstable, and photolabile. Vibrational data have been reported for mutant SOR (E47A) peroxos generated via the addition of H_2O_2 .^{2b,f,g} Whether these are identical to the catalytic SOR intermediate remains to be determined. Although a few well-characterized synthetic nitrogen-ligated iron–peroxos have been reported,^{4a,c} there is a paucity of thiolate-ligated analogues.^{4b} Since a thiolate is likely to influence the correlation between peroxide binding mode, vibrational parameters, and spin–state, synthetic thiolate–ligated peroxos are needed to provide benchmark parameters. Prior to the work reported herein, *cis* thiolate-ligated $[Fe^{III}(S^{Me2}N_4(tren))(OOH)]^+$ (**1**),^{4b} was the only reported example of a synthetic thiolate–ligated Fe^{III} –peroxo.

In situ deprotection and deprotonation of the new macrocyclic ligand cyclam–PrS–Ac·4HCl, afforded $[Fe^{II}(cyclam–PrS)](BPh_4)$ (**2**) upon the addition of $FeCl_2$ and NaBPh₄. Single crystals were grown from pentane/THF at –30 °C. As shown in the ORTEP (Figure 1), the Fe^{2+} ion of **2** is ligated by three secondary amines, one tertiary amine, and a tethered apical thiolate in a square pyramidal geometry ($\tau = 0.13$)⁵ resembling that of SOR. A related tertiary amine cyclam complex $[Fe^{II}(Me_3–cyclam–EtS)]^+$ (**3**) was recently reported⁶ that reacts with H_2O_2 to afford an $Fe(IV)=O$.⁷ Like the SOR active site, **2** is high spin ($S = 2$; $\mu_{eff} = 5.03$ BM (MeCN); 4.91 BM (solid)). The Fe–S bond length in **2** (2.286(1) Å) falls in the usual range for synthetic Fe(II)–thiolates,^{6,8} but is slightly shorter than that of SOR (Fe–S = 2.4 Å), the cysteinate sulfur of which is H-bonded to the protein backbone.^{2a,c}

Thiolate–ligated **2** reacts rapidly with $O_2^{\bullet -}$ (18-crown-6- K^+ salt) in CH_2Cl_2 at –78 °C to afford a metastable burgundy intermediate, as soon as a proton donor (MeOH; 82 equiv) is added.

This intermediate is high-spin ($g = 7.72, 5.40, 4.15$), and displays an absorption band at $\lambda_{\text{max}} = 530(1350)$ nm. Resonance Raman shows $\nu_{\text{O-O}}$, $\nu_{\text{Fe-O}}$, and $\nu_{\text{Fe-S}}$ stretches at 891 cm^{-1} (Fermi doublet), 419 cm^{-1} , and 352 cm^{-1} , respectively (Figure 2). K^{18}O_2 (50% enriched; ICON) causes the $\nu_{\text{O-O}}$ and $\nu_{\text{Fe-O}}$ to shift to 856 cm^{-1} and 400 cm^{-1} , respectively, and addition of D^+ (*ie*, MeOD) causes the Fermi doublet to collapse. These data are consistent with the formation of an Fe-hydroperoxo species, $[\text{Fe}^{\text{III}}(\text{cyclam-PrS})(\text{OOH})]^+$ (**4**), via the proton-dependent oxidative addition of superoxide to **2**. No reaction occurs in the absence of a proton donor, and O_2^- does not convert to H_2O_2 in the absence of **2**, under the conditions examined (-78°C , 82 equiv MeOH). Intermediate **4** represents the first example of a synthetic *trans* thiolate-ligated Fe^{III} -peroxo SOR intermediate analogue, the characterization of which provides important benchmark parameters.

The $\nu_{\text{Fe-O}}$ stretch of **4** is significantly lower than all other reported synthetic iron peroxides (range: $450\text{--}639\text{ cm}^{-1}$),^{4a} but compares well with that of the only reported SOR peroxo (438 cm^{-1}).^{2a,e} The $\nu_{\text{O-O}}$ stretch (891 cm^{-1}) is unusually high (reported range: $820\text{--}860\text{ cm}^{-1}$).^{4a} The DFT optimized structure of **4** minimizes with Fe-S and Fe-O distances of $2.36\text{ \AA}$ and $1.95\text{ \AA}$, respectively, and a protonated peroxo O-O distance of $1.44\text{ \AA}$. This Fe-O(peroxo) distance is significantly longer than the few reported Fe-(η^1 -OOH) structures ($1.76\text{--}1.86\text{ \AA}$)^{4a} reflecting the *trans* influence of the thiolate sulfur. The calculated $\nu_{\text{Fe-S}}$ (345 cm^{-1}), $\nu_{\text{Fe-O}}$ (400 cm^{-1}) and $\nu_{\text{O-O}}$ (933 cm^{-1}) stretches are in reasonable agreement with the experimental data. When the thiolate is replaced with an amine, or alkoxide, *trans* to the peroxo,⁹ then the calculated $\nu_{\text{Fe-O}}$ (495 cm^{-1} and 420 cm^{-1} , respectively) is considerably higher. These vibrational data, along with the calculated force constant ($k_{\text{Fe-O}} = 1.20\text{ mdynes/cm}^2$ for **4** vs reported range = $2.2\text{--}2.1\text{ mdynes/cm}^2$),^{4a} indicate that the Fe-O(peroxide) bond is significantly weakened upon the introduction of a *trans* thiolate into the coordination sphere.

Addition of HOAc to metastable **4** at -78°C releases H_2O_2 (as detected using an amplex red assay), and cleanly affords a new aqua blue species $\lambda_{\text{max}} = 604(1350)$ nm (Figure 3). When this reaction is monitored by EPR, the high-spin signal associated with **4** is replaced with a new low-spin signal at $g = 2.37, 2.30, 1.89$. The $\nu_{\text{O-O}}$ and $\nu_{\text{Fe-O}}$ stretches disappear in the rRaman spectrum, and new stretches are observed at $339, 409$, and 421 cm^{-1} . Although this aqua blue species proved too unstable to isolate, it was unambiguously identified by ESI-mass spectrometry as acetate-bound $[\text{Fe}^{\text{III}}(\text{cyclam-PrS})(\text{OAc})]^+$ (**5**), a model for Glu-bound SOR.

Addition of a sacrificial reductant (Cp_2Co) to **5** at low temperatures (-78°C) regenerates **2**, which then reacts with a second equiv of O_2^- to re-afford peroxo **4**. Addition of a second equiv of HOAc releases H_2O_2 (Figure 4), thereby mimicking the proposed SOR catalytic cycle involving glutamic acid,^{2d,e,i} and demonstrating that reduction of O_2^- by **2** is catalytic. Thus far, five turnovers have been achieved.

The thiolate ligand and its *trans* positioning relative to the substrate appear to contribute significantly to the function of our biomimetic catalyst. First, the pendent thiolate arm of **2** causes the redox potential to shift anodically by $+480\text{ mV}$ relative to $[\text{Fe}^{\text{II}}(\text{cyclam})(\text{MeCN})_2]$ (from $+700\text{ mV}$ to $+220\text{ mV}$ vs SCE), making it better suited to promote superoxide reduction. Second, the *trans* thiolate changes the spin-state from $S = 1/2$ ^{4b} to $S = 5/2$: the majority of nitrogen-ligated $\text{Fe}(\text{III})\text{-OOH}$'s are $S = 1/2$,^{4a} as is *cis*-thiolate ligated **1**.^{4b} Third, the thiolate dramatically shifts the $\nu_{\text{Fe-O}}$ stretch and decreases the $k_{\text{Fe-O}}$ force constant well-below all other reported iron peroxides.^{4a} Peroxo **4** partially converts to methoxide-bound $[\text{Fe}^{\text{III}}(\text{cyclam-PrS})(\text{OMe})]^+$ (**6**; $g = 2.34, 2.26, 1.95$; $\nu_{\text{Fe-S}} = 357\text{ cm}^{-1}$) within minutes at -78°C , whereas *cis*-ligated peroxo **1** takes hours ($t_{1/2} = 63.9\text{ hrs}$) to convert to $[\text{Fe}^{\text{III}}(\text{S}^{\text{Me}_2}\text{N}_4(\text{tren}))(\text{OMe})]^+$ under the same conditions. Methoxide-bound **6** was identified via its independent synthesis involving Cp_2Fe^+ oxidation of **2** in MeOH, in the presence of $i\text{Pr}_2\text{EtN}$.

In conclusion, the data described herein indicates that like the enzyme, SOR intermediate-analogue **4** is better suited to promote Fe–O, as opposed to O–O, bond cleavage. This is in contrast to P450 and its analogue **3**. Kinetics studies, and studies aimed at determining the pK_a of the proximal and distal peroxo oxygens of **4** are currently underway.

Supplementary Material

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Figure 1. ORTEP of $[\text{Fe}^{\text{II}}(\text{cyclam-PrS})]^+$ (**2**). Selected bond lengths ($\text{\AA}$): Fe–S(1), 2.286(1); Fe–N(1), 2.181(4); Fe–N(2,3,4)_{avg}, 2.16(2).

Figure 2. rRaman spectra of **4** generated from $^{16}\text{O}_2^-$ (blue), $^{18}\text{O}_2^-$ (red), and “decayed” product (dashed black) (571 nm excitation @ 183 K in THF/MeOH (upper panel); @ 77K in CH_2Cl_2 /THF/MeOH (lower panel).

Figure 3. Conversion of peroxo-bound **4** (0.5 mM in CH₂Cl₂) to acetate-bound [Fe^{III}(cyclam-PrS)(OAc)]⁺ (**5**) via the addition of HOAc (0.1 equivalent every two minutes) at -78 °C.

Figure 4. The catalytic cycle involving $[\text{Fe}^{\text{II}}(\text{cyclam-PrS})]^+$ (**2**) induced superoxide (O_2^-) reduction