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Use of a Metallopeptide Based Mimic Provides Evidence for a Proton Coupled Electron Transfer Mechanism For Superoxide Reduction By Nickel Containing Superoxide Dismutase**

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Superoxide ($O_2^{\bullet-}$) is a highly toxic reactive oxygen species that can induce cellular death at subnanomolar concentrations.^[1] Because $O_2^{\bullet-}$ is an unavoidable fact of aerobic life, aerobic organisms have evolved superoxide detoxification systems, the most prevalent of which are the superoxide dismutases (SODs).^[1,2] All known SODs have redox-active metal cofactors and detoxify superoxide through a ping-pong type mechanism:

where M^{red} and M^{ox} are the reduced and oxidized forms of the metalloenzyme's metal cofactor. The most recently discovered SOD contains nickel at its active-site (NiSOD), is found in several soil and aquatic bacteria, and operates by cycling between the reduced Ni(II) and oxidized Ni(III) oxidation states.^[2f,3] In its reduced state, square planar Ni(II) is ligated by two *cis*-cysteinate sulfur atoms (Cys2 and Cys6), an amidate nitrogen atom from Cys2 and the *N*-terminal amine nitrogen atom from His1 (Scheme 1).^[4] Oxidation to Ni(III) yields a square pyramidal structure via the ligation of the His1 imidazole δ -nitrogen to Ni. Thus, all of the ligating residues to Ni are contained within the first six residues of the NiSOD primary sequence.

We,^[3d,5] and others,^[6] have exploited this convenience of nature to generate active NiSOD biomimetics by utilizing peptides containing the first 6-12 residues of the NiSOD sequence. Previously we demonstrated that one of these metallopeptide based NiSOD mimics, {Ni(SOD^{M1}H(1)H^{Me})} (Ni^{M1}; SOD^{M1}H(1)H^{Me} = H₂N-H^{Me}CDLP CGVYD PA; H^{Me} = ^{*e*}*N*-methyl-histidine), is isolable and stable at room temperature in both the Ni(II) (Ni(II)^{M1}) and Ni(III) (Ni(III)^{M1}) oxidation states.^[5a] Ni^{M1} also performs catalytic superoxide disproportionation at reasonably slow velocities ($k = 6(1) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$; pH = 8.0) making it an ideal candidate for detailed mechanistic study.

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The mechanism by which NiSOD disproportionates superoxide has not been explored in any detail experimentally. Besides issues involving inner-sphere vs. outer-sphere $\text{O}_2^{\bullet-}$ oxidation/reduction, one mechanistic point that has not been explored is the role of proton donation in the superoxide reduction half-reaction (1a). Herein, we present evidence using Ni^{M1} that superoxide disproportionation catalyzed by NiSOD can occur through a proton-coupled electron transfer (PCET) type mechanism.

We first turned our attention to the superoxide disproportionation kinetics catalyzed by Ni^{M1} using stopped-flow techniques as previously described.^[5a,7] The reaction was performed at pH 8.0 to suppress the $\text{O}_2^{\bullet-}$ self-disproportionation reaction. At 25.0 °C a $k_{\text{cat}} = 6.1(2) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ was obtained, in line with what was previously observed (Figure 1). Performing the reaction in D_2O (pD = 8.0)^[8] resulted in a substantial solvent KIE; the D_2O $k_{\text{cat}} = 3.1(2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, which translates into a solvent KIE of 20(4). Such a large room temperature KIE is suggestive of a PCET event with a substantial quantum mechanical tunneling component. We note that we measure a superoxide self-disproportionation solvent KIE of 1.2(1) under these conditions.

To determine if a quantum mechanical tunneling event is a tenable hypothesis we examined the temperature dependence of k_{cat} on the Ni^{M1} catalyzed disproportionation of superoxide. We were able to accurately determine rate constants over the temperature range of 2.0 – 75.0 °C. The Arrhenius plot of superoxide disproportionation is relatively flat, but none-the-less displays a slight concave appearance (Figure 2). A traditional Arrhenius analysis suggests a near barrierless reaction ($E_a = 0.60(1) \text{ cal mole}^{-1}$; $A = 6.45(3) \times 10^6 \text{ s}^{-1}$). These values are wholly inconsistent with estimated and measured SOD energetics.^[10] What the variable temperature kinetics data are consistent with is that SOD catalysis facilitated by Ni^{M1} is operating via a tunneling mechanism.^[11]

Based on the above experiments it seems reasonable to propose that Ni^{M1} can facilitate the reduction of $\text{O}_2^{\bullet-}$ through a PCET reaction. We should therefore be able to facilitate the reduction of $\text{Ni}(\text{III})^{\text{M1}}$ with H-atom donors possessing moderate X-H bond dissociation free energies (BDFEs). $\text{Ni}(\text{III})^{\text{M1}}$ was thus subjected to a series of reactions at pH 8. We could effect the reduction of $\text{Ni}(\text{III})^{\text{M1}}$ to $\text{Ni}(\text{II})^{\text{M1}}$ using ascorbate (BDFE = 73.6 kcal mole⁻¹)^[12] and TEMPO-H (BDFE = 71.0 kcal mole⁻¹).^[12] Furthermore, $\text{Ni}(\text{III})^{\text{M1}}$ could be regenerated by adding TEMPO• to $\text{Ni}(\text{II})^{\text{M1}}$. In contrast, we could not facilitate the reduction of $\text{Ni}(\text{III})^{\text{M1}}$ utilizing reagents with slightly stronger X-H bonds, such as 1,4-dihydroquinone (first BDFE = 81.5 kcal mole⁻¹)^[12] and hydrazine (first BDFE = 83.4 kcal mole⁻¹).^[12] This would suggest that 1) there is a hydrogen atom acceptor site within $\text{Ni}(\text{III})^{\text{M1}}$ that allows for the generation of $\text{Ni}(\text{II})^{\text{M1}}\text{-H}$ (i.e. protonated $\text{Ni}(\text{II})^{\text{M1}}$), and 2) that the $\text{Ni}(\text{II})^{\text{M1}}\text{-H}$ BDFE is between 70 to 80 kcal mole⁻¹.

To better estimate the $\text{Ni}(\text{II})^{\text{M1}}\text{-H}$ BDFE an equilibrium reaction between TEMPO•/TEMPO-H and $\text{Ni}(\text{II})^{\text{M1}}\text{-H}/\text{Ni}(\text{III})^{\text{M1}}$ was established (Scheme 2). By varying the concentration of TEMPO• and $\text{Ni}(\text{II})^{\text{M1}}\text{-H}$ and allowing the reaction to come to equilibrium we could calculate the equilibrium constant, K , for the reaction. At 25.0 °C we obtain a $K = 26.5(8)$ for the $\text{Ni}(\text{III})^{\text{M1}} + \text{TEMPO-H} \rightleftharpoons \text{Ni}(\text{II})^{\text{M1}}\text{-H} + \text{TEMPO}\bullet$ equilibrium process, which translates into a $\Delta G = -1.9(7) \text{ kcal mole}^{-1}$. We can thus estimate a BDFE for the $\text{Ni}(\text{II})^{\text{M1}}\text{-H}$ bond of 73(1) kcal mole⁻¹. The measured BDFE is in line with the calculated BDFE based on the $\text{Ni}(\text{II})^{\text{M1}}\text{-H}$ $\text{p}K_a$ and the previously determined redox potential of Ni^{M1} (440 mV vs. NHE).^[5a] Measuring differences in the ligand-field bands of $\text{Ni}(\text{II})^{\text{M1}}\text{-H}/\text{Ni}(\text{II})^{\text{M1}}$ as a function of pH yields a $\text{Ni}(\text{II})^{\text{M1}}\text{-H}$ $\text{p}K_a$ of ~8.2. Application of a modified Bordwell equation^[12] yields a $\text{Ni}(\text{II})^{\text{M1}}\text{-H}$ BDFE of ~79 kcal mole⁻¹, which is in good agreement with the above measurement considering the associated errors in the Ni^{M1} redox potential measurement.^[5a] Both of these measurements are in line with the superoxide

reduction process; the deprotonated product, HO_2^- , has an O-H BDFE of 81.6 kcal mole⁻¹.^[12] For $\text{Ni(II)}^{\text{M1}}\text{-H}$ to effectively facilitate the reduction of $\text{O}_2^{\bullet-}$ through a PCET reaction it must have a BDFE less than the HO_2^- O-H BDFE. Otherwise the reaction would be overall uphill and not thermodynamically viable.

If there is an abstractable H-atom within $\text{Ni(II)}^{\text{M1}}\text{-H}$, then there must be a well defined protonation site near the Ni(II) active site. An initial clue to the location of the well-defined protonation site within $\text{Ni(II)}^{\text{M1}}\text{-H}$ was provided by Ni K-edge X-ray absorption spectroscopy (XAS) recorded at elevated pH. Previously we demonstrated that at pH 7.4 $\text{Ni(II)}^{\text{M1}}\text{-H}$ has an average Ni-S bond length of 2.18 Å.^[5a] At pH 9.5 $\text{Ni(II)}^{\text{M1}}$ has an average Ni-S bond length of 2.20 Å. This increase in bond length is consistent with a thiolate deprotonation event.^[13a] Although counterintuitive, the origin of the contraction of the Ni-S bond in square planar $\text{Ni(II)N}_2\text{S}_2$ complexes upon protonation or hydrogen-bond formation is well known.^[13] Protonation of the coordinated S *via* a filled S(p)-type lone-pair reduces unfavorable filled-filled Ni(π)/S(π) interactions leading to a bond contraction. Thus, the elongation of the Ni-S bond at high pH is consistent with cysteinate deprotonation.

A protonated Ni-S(H)-Cys bond is also consistent with room temperature S K-edge XAS performed at pH 7.4 vs. 9.5. At pH 9.5 the S K-edge X-ray absorption spectrum of $\text{Ni(II)}^{\text{M1}}$ is consistent with a thiolate ligated Ni(II) complex (Figure 3).^[14] The main S(1s) $\rightarrow$ S(C-S σ^*) transition occurs at 2472.6(1) eV. Prior to the S(1s) $\rightarrow$ S(C-S σ^*) transition there is a prominent pre-edge feature corresponding to the nominal S(1s) $\rightarrow$ Ni(3d)/S(σ)* transition at 2470.4(1) eV. Similarly, the pH 7.4 S K-edge spectrum of oxidized $\text{Ni(III)}^{\text{M1}}$ is consistent with a thiolate ligated Ni(III) complex; the main S(1s) $\rightarrow$ S(C-S σ^*) transition occurs at 2473.0(1) eV while the S(1s) $\rightarrow$ Ni(3d)/S transitions at $\sim$ 2470 eV is broad, displays asymmetry and is more intense. The change in intensity and peak shape is consistent with a) the increase in number of holes in the Ni(3d) manifold from 2 to 3 upon oxidation and b) the fact that a lower-energy Ni(3d)/S(π)* type orbital is now only partially filled and will accept an electron upon promotion from the S(1s) orbital. These two spectra can be contrasted with the pH 7.4 S K-edge X-ray absorption spectrum of $\text{Ni(II)}^{\text{M1}}\text{-H}$. First, there is a broadening of the main S(1s) $\rightarrow$ S(C-S σ^*) transition. Second, and more obvious, the pre-edge feature virtually disappears into the baseline. There are three conclusions that can be drawn from these results. One is that the pH 7.4 form of $\text{Ni(II)}^{\text{M1}}\text{-H}$ is protonated at one (or both) of the coordinated cysteinates.^[15] The second conclusion is that at pH 9.5 the coordinated cysteinate(s) become deprotonated. The last conclusion is that $\text{Ni(III)}^{\text{M1}}$ does not contain a protonated coordinated cysteinate at low pH.

It seems reasonable to propose that the source of the formal H-atom in reduced $\text{Ni(II)}^{\text{M1}}\text{-H}$ is a protonated Ni(II)-S(H)Cys moiety. One may expect that the $\text{p}K_a$ of a coordinated thiolate would be rather low and inconsistent with this formulation. However, we suspect that the *cis*-Cys moiety, or possibly the coordinated amine *cis* to the Cys residue, may substantially raise the $\text{p}K_a$ of the Ni(II)-S(H)Cys moiety higher than expected through a cooperative effect as seen in 1,8-bis(dimethylamino)naphthalene (proton sponge), for example. Other structural factors in metalloprotein microenvironment about Ni(II) center may also be contributing to the elevated Ni(II)-S(H)Cys $\text{p}K_a$.

In summary, we have provided evidence that superoxide disproportionation catalyzed by Ni^{M1} occurs through a rate-limiting PCET mechanism with a significant tunneling component. It appears that the PCET step occurs during the reduction of $\text{O}_2^{\bullet-}$ by protonated $\text{Ni(II)}^{\text{M1}}\text{-H}$, and the formal H-atom is in the form of a coordinated Ni(II)-S(H)^{Cys} moiety (Scheme 3). This proposal of a PCET mechanism during superoxide reduction appears to be unique amongst the SODs, save a recent study of Cu/Zn-SOD (*vide infra*).

Computationally, there has been one hybrid DFT study that has explicitly invoked a PCET event during the NiSOD catalyzed reduction of $O_2^{\bullet-}$.^[16] In that study the transferred “H-atom” is in the form of a Ni-S(H)-Cys moiety. Our findings are also consistent with the limited experimental data available concerning NiSOD itself, as well as some more recent studies on Cu/Zn SOD. First, there is strong evidence from S K-edge X-ray absorption spectroscopy that reduced NiSOD itself contains a protonated Ni(II)-S(H)Cys moiety while the cysteinate is deprotonated upon NiSOD oxidation.^[15] Second, the crystal structure of NiSOD reveals an unusual structural feature; the Ni-S bond *trans* to the amidate nitrogen is shorter than the Ni-S bond *trans* to the amine nitrogen.^[4] This is the opposite of what one would expect based on the synthetic Ni(II)N₂S₂ models with amine vs. amidate coordination.^[13a,b,17] It therefore seems reasonable to suggest that the Ni-S^{Cys} moiety *trans* to the amidate is indeed protonated in NiSOD. Analysis of the pH dependence of SOD catalysis by NiSOD itself shows that k_{cat} is pH independent over the range of 6–8 and then falls off dramatically above 8.0, which is suggestive of the strong coupling of the proton-transfer event with the redox chemistry of $O_2^{\bullet-}$.^[3c] Lastly, we note that at least one other SOD appears to operate through a rate-limiting PT event. A recent natural abundance ¹⁸O/¹⁶O KIE study by Roth and Smirnov provided evidence that an initial rate limiting proton transfer event is occurring in the reduction of $O_2^{\bullet-}$ by Cu/Zn SOD under high pH conditions.^[10a] We are currently in the process of determining the extent to which H-atom transfer from a M^{red}-S(H)^{Cys} moiety is relevant in NiSOD itself as well as other metalloenzymes, such as [Ni,Fe]-hydrogenase.^[18]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.
NiSOD (R = H) and Ni^{M1} (R = CH_3) active sites.

Figure 1.

Superoxide decay kinetics in the presence and absence of Ni^{M1} at pH/pD 8.0 (25.0 °C). Data is represented by the solid lines and the second order fits are represented by the dashed lines. The gradual increase in absorbance at long time lengths is related to the H_2O_2 initiated decomposition of Ni^{M1} taking place after the $O_2^{\bullet-}$ disproportionation is complete.

Figure 2. Arrhenius plot of $\{\text{Ni}(\text{SOD}^{\text{M1}}\text{H}(1)\text{H}^{\text{Me}})\}$ catalyzed decay of superoxide at pH/pD 8.0. The main plot depicts the data collected in H_2O , the inset depicts the data collected in D_2O . The error bars are the size of the dots or smaller. The trend lines were generated using a modified Bell Tunneling expression^[11a] (see supporting information).

Scheme 2.

Equilibrium reaction between $\text{Ni(III)}^{\text{M1}} + \text{TEMPO-H}$ and $\text{Ni(II)}^{\text{M1-H}} + \text{TEMPO}^\bullet$

Figure 3. Sulfur K-edge XAS spectra of **Ni(II)^{M1}-H** recorded at pH 7.4 (solid), **Ni(II)^{M1}** recorded at pH 9.5 (dotted), and **Ni(III)^{M1}** recorded at pH 7.4 (dashed).

Scheme 3.

Proposed mechanism of $\text{O}_2^{\bullet-}$ reduction by $\{\text{Ni}^{\text{II}}(\text{SOD}^{\text{M1}}\text{H}(1)\text{H}^{\text{Me}})\text{-H}\}$ and NiSOD .