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A Nickel Superoxide Dismutase Maquette That Reproduces the Spectroscopic and Functional Properties of the Metalloenzyme

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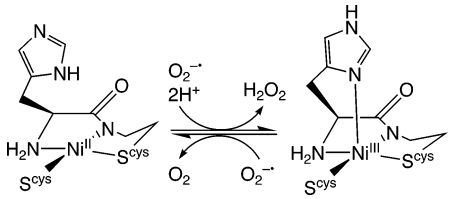
Described herein is a nickel superoxide dismutase (NiSOD) maquette ([Ni(SOD^{M1})]) based on the first 12 residues from the N-terminal sequence of *Streptomyces coelicolor* NiSOD. The apopeptide (SOD^{M1}) was prepared by standard solid-phase Fmoc peptide synthesis. SOD^{M1} will readily coordinate Ni^{II} in a 1:1 ratio in slightly basic aqueous sodium phosphate buffer (0.1 M; pH = 7.2) forming a lightly colored beige/pink solution. Unlike NiSOD, which is isolated as a 1:1 mixture of oxidized (Ni^{III}) and reduced (Ni^{II}) forms, [Ni(SOD^{M1})] can only be isolated in the Ni^{II} oxidation state. The UV/vis, X-ray absorption, and CD spectra of [Ni^{II}(SOD^{M1})] correspond well with those reported for the reduced form of NiSOD. Despite the fact that [Ni^{III}(SOD^{M1})] is not isolable, [Ni(SOD^{M1})] has an appropriate redox potential to act as an SOD ($E_{1/2}$ = 0.70(2) V vs Ag/AgCl) and in fact will catalytically disproportionate >40 000 equiv of KO_2.

Aerobic organisms must utilize detoxification pathways to protect themselves against oxidative damage resulting from exposure to reactive oxygen species (ROS), which are produced from the adventitious reduction of dioxygen.[1,2] One ROS, superoxide ($O_2^{\bullet-}$), is usually destroyed by metalloenzymes called superoxide dismutases (SODs).[1,3,4] SODs catalyze the disproportionation of $O_2^{\bullet-}$ into dioxygen and hydrogen peroxide and, until recently, were thought to fall into one of three classes: Fe, Mn, and Cu/Zn SODs.[4] In the late 1990s, a fourth class of SOD containing Ni (NiSOD) was isolated from several *Streptomyces* species and cyanobacteria.[5,6] NiSODs are mononuclear Ni-containing metalloenzymes that catalyze $O_2^{\bullet-}$ disproportionation by cycling between the Ni^{II} and Ni^{III} oxidation states.[7−9] Crystallographic[7,8] and spectroscopic studies[9,10] have elucidated the primary coordination sphere of NiSOD in both its oxidized and reduced states. In the reduced state, the Ni^{II} center is contained in a square-planar geometry ligated by two *cis*-cysteinate sulfurs, one amide nitrogen derived from the peptide backbone, and the N-terminus amine nitrogen from His(1). Oxidation of NiSOD to the Ni^{III} oxidation state produces a structural change about the metal center; the N-terminal histidine imidazole coordinates to Ni^{III} in the axial position (Scheme 1).

Scheme 1



Despite the fact that the structures of reduced and oxidized NiSOD have been elucidated, a number of questions concerning both the detailed mechanism of $O_2^{\bullet-}$ disproportionation and how secondary coordination-sphere residues influence reactivity remain unanswered.[7] To better understand the fundamental chemistries of NiSODs, we have prepared maquettes that closely mimic the spectroscopic and structural properties of the parent metalloenzyme. Metalloprotein maquettes are small metallopeptides that replicate some aspect of their biological inspiration[11] and can offer many of the advantages of traditional metalloenzyme synthetic models[12,13] while still utilizing a biological scaffold. Com-

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municated herein are results from our first-generation NiSOD maquette, [Ni(SOD^{M1})].

[Ni(SOD^{M1})] is based on the first 12 residues from the N-terminus of the active form of *S. coelicolor* NiSOD (H_2N−HCDLPCGVYDPA−COOH).[7] The apopeptide (SOD^{M1}) was synthesized via standard Fmoc solid-phase synthetic methods on Wang resin using HOBt/DIC coupling chemistry. SOD^{M1} was cleaved from the resin, washed with freshly distilled diethyl ether, incubated at 45 °C with β-mercaptoethanol, and purified by preparative reverse-phase HPLC. Ni^{II} was then incorporated into the apopeptide using $NiCl_2$ under anaerobic conditions forming [Ni(SOD^{M1})].[14] Incorporation of Ni^{II} into SOD^{M1} is highly pH dependent. When the pH is less than ~6, Ni^{II} will not coordinate to the peptide even when a large excess of $NiCl_2$ is utilized. However, at pH = 7.2 (0.1 M sodium phosphate buffer) Ni^{II} will coordinate to SOD^{M1} in an ~1:1 ratio (based on spectrophotometric titrations), forming a weakly colored beige-pink solution. [Ni(SOD^{M1})] exists in solution as a monomer as assessed by gel permeation chromatography, while CD studies demonstrate that the peptide structure of [Ni(SOD^{M1})] is best described as a random coil with some β-turn character.[15]

Unlike NiSOD, which is isolated as a 1:1 mixture of Ni^{II}/Ni^{III} oxidation states,[7] [Ni(SOD^{M1})] is obtained exclusively in the Ni^{II} oxidation state even when isolated in air. This is evident from the 1D ^1H NMR spectrum of the metallopeptide, which does not display any paramagnetically shifted peaks or line-broadening, suggesting that paramagnetic Ni^{III} is not present. Attempts were made to produce and isolate the Ni^{III} form of [Ni(SOD^{M1})] with no success. Not surprisingly, the addition of dilute H_2O_2 or Oxone, which can oxidize coordinated thiolate-sulfurs,[16] leads to the rapid and complete decomposition of [Ni(SOD^{M1})]. We also found that the addition of 1 equiv of ceric ammonium nitrate or [Fe^{III}(bipy)$_3$]Cl_3 to anaerobic solutions of [Ni^{II}(SOD^{M1})] leads to the decomposition of the metallopeptide. In addition, we observed that solutions of [Ni^{II}(SOD^{M1})] will decompose if exposed to air over the course of several hours. In all cases, the decomposition products are insoluble high-molecular-weight polymers.

Reduced [Ni^{II}(SOD^{M1})] displays relatively weak features in the UV/vis spectrum (Figure 1). These features do not change when dithionite is added to solutions of [Ni^{II}(SOD^{M1})], further supporting the Ni^{II} oxidation state. The most distinct feature in the UV/vis spectrum is a broad transition with a well-defined maximum at 21 800 cm^{-1} (458 nm; ϵ = 510 M^{-1} cm^{-1}) and a shoulder at 18 100 cm^{-1} (552 nm; ϵ = 240 M^{-1} cm^{-1}). At higher energy, there is a shoulder observed at 29 700 cm^{-1} (337 nm; ϵ = 1060 M^{-1} cm^{-1}) just before the onset of end absorbance. All three of these features correspond to those observed in reduced NiSOD.[10] The corresponding CD spectrum of [Ni^{II}(SOD^{M1})] helps to

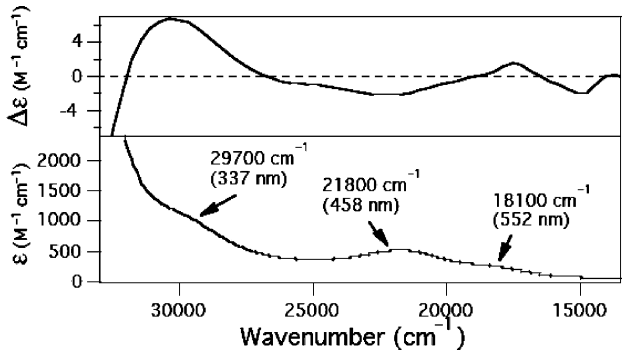

Figure 1. UV/vis (bottom) and CD spectra (top) of [Ni(SOD^{M1})] (20 °C, 0.1 M sodium phosphate buffer, pH = 7.2).

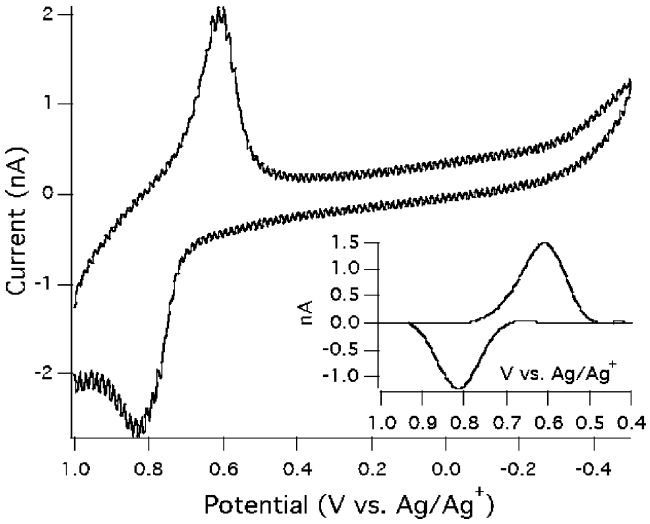

Figure 2. Cyclic voltammogram of [Ni(SOD^{M1})] recorded as a "protein thin film" on a carbon fiber ultramicroelectrode.[14] The inset depicts the data with the background subtracted.

resolve many of these features (Figure 1), most of which correspond well with reduced NiSOD.[10] Most notable is the negative signed feature observed at 21 800 cm^{-1} ($\Delta\epsilon_{max}$ = −2.2 M^{-1} cm^{-1}), which is also observed in the reduced metalloenzyme. One feature that we observe in [Ni^{II}(SOD^{M1})] that was not observed in reduced NiSOD is a weak band at 15 100 cm^{-1} (ϵ = 70 M^{-1} cm^{-1}), presumably a ligand-field transition, which is well resolved in the CD spectrum ($\Delta\epsilon_{max}$ = −2.0 M^{-1} cm^{-1}).

The Ni K-edge X-ray absorption spectrum of [Ni^{II}(SOD^{M1})] is also consistent with that reported for reduced NiSOD.[9] The pre-edge region displays a prominent peak at 8337.4(2) eV corresponding to a 1s → $4p_z$ transition and a weak peak at 8332.9(4) eV corresponding to a 1s → 3d transition. These are both indicative of square planar Ni^{II}. Best fits to the EXAFS region revealed two Ni−N scatterers at 1.93(2) Å and two Ni−S scatterers at 2.180(2) Å, which compares well with those reported for reduced NiSOD (Ni−N_{ave}, 1.89 Å; Ni−S_{ave}, 2.18 Å).[7−9]

The electrochemical behavior of [Ni(SOD^{M1})] was measured using "protein thin-film voltammetry" techniques[17] on

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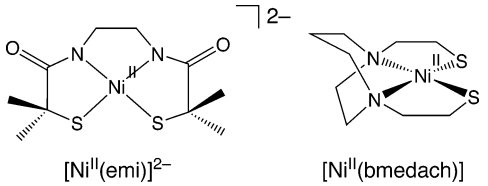
a carbon fiber ultramicroelectrode. [Ni(SOD^{M1})] has an E_{pc} = 0.61(1) V and an E_{pa} = 0.80(2) V vs Ag/Ag$^+$. We note that the relatively large peak separation (0.19 vs ~0.02 V typically obtained for fully reversible reactions recorded under similar conditions) observed for [Ni(SOD^{M1})] is indicative of kinetic limitations to e$^-$ transfer (i.e., a rate-limiting change in coordination environment occurrs upon e$^-$ transfer). This is unexpected considering the rapid rate of $O_2^{\bullet-}$ disproportionation observed for NiSOD[9] and may have implications concerning the mechanism of $O_2^{\bullet-}$ disproportionation in the metalloenzyme and the maquette. Although the midpoint potential observed for [Ni(SOD^{M1})] is ~0.4 V more positive than is usually observed for SODs,[4] it is not unprecedented for thiolate-ligated Ni complexes.[19,20] Furthermore, the [Ni(SOD^{M1})] redox potential of 0.70(2) V vs Ag/Ag$^+$ suggests that it may be capable of behaving as an SOD ($E_{ox}^{O2\bullet-}$ = 0.04 V; $E_{red}^{O2\bullet-}$ = 1.09 V vs Ag/Ag$^+$).

In light of our electrochemical data, we investigated the ability of [Ni(SOD^{M1})] to disproportionate superoxide in aqueous solutions. We first examined the superoxide decomposition products following the addition KO_2 to solutions of [Ni^{II}(SOD^{M1})]. Addition of solid KO_2 to [Ni^{II}(SOD^{M1})] results in the formation of bubbles, indicating the production of dioxygen. Once bubbling had ceased, we assayed the solution for H_2O_2 content. For every mole of KO_2 added to a solution of [Ni^{II}(SOD^{M1})] ~0.5 mol of H_2O_2 are produced, indicating that [Ni(SOD^{M1})] might be behaving as an SOD (as opposed to a superoxide reductase). To address if [Ni(SOD^{M1})] is truly catalyzing the disproportionation of $O_2^{\bullet-}$ we utilized the *para*-nitro blue tetrazolium chloride (NBT)/formazan assay.[5a,14]

NBT reacts with $O_2^{\bullet-}$ forming the blue pigment formazan ($\lambda_{max} \approx$ 580 nm (~35 000 M^{-1} cm^{-1})). The addition of 1400 equiv[18] of KO_2 to a 33 μM NBT solution produced an intense blue color with a band centered at 580 nm in the UV/vis. In contrast, when the same reaction is performed with [Ni(SOD^{M1})] (10 μM) present, formazan is not produced. This suggests that [Ni(SOD^{M1}) is behaving as an SOD, protecting NBT from oxidative damage. The fact that [Ni(SOD^{M1})] displays SOD activity is surprising in light of our failed attempts at preparing the Ni^{III} form of the maquette and indicates that [Ni^{III}(SOD^{M1})] is at least quasi-stable. Preliminary UV/vis studies indicate that, although [Ni^{II}(SOD^{M1})] will rapidly decompose following the addition of KO_2, it does not completely decompose until ~90 s has passed. This indicates that [Ni(SOD^{M1})] will survive in solution long enough to catalytically disproportionate $O_2^{\bullet-}$.

Scheme 2



[Ni^{II}(emi)]$^{2-}$ [Ni^{II}(bmedach)]

The addition of 2800 equiv[18] of KO_2 to a [Ni(SOD^{M1})]/NBT solution produces a faint blue color (and a weak band at 580 nm), indicating that [Ni(SOD^{M1})] is losing SOD activity when exposed to a large excess of KO_2. However, it is not until ~16 500 equiv[18] of KO_2 are added to the solution that [Ni(SOD^{M1})] is overwhelmed, as noted by the full formation of formazan from NBT. This suggests that [Ni(SOD^{M1})] is capable of disproportionating up to 40 000 equiv of $O_2^{\bullet-}$ before it can no longer offer NBT protection from oxidative damage. We note that neither the apopeptide SOD^{M1} nor $NiCl_2$ will offer NBT protection from KO_2.

We also investigated the ability of two NiN_2S_2 complexes ([Ni^{II}(bmedach)] (**1**) and [Ni^{II}(emi)]$(Et_4N)_2$ (**2**, Scheme 2)[19,20] to protect NBT from KO_2. Neither **1** nor **2** displays SOD activity under the conditions employed in the above experiments; NBT was rapidly and fully converted to formazan after the addition of 1400 equiv of KO_2[18] in each case. The differences in the $O_2^{\bullet-}$ reactivity between [Ni(SOD^{M1})] and the two NiN_2S_2 complexes investigated can be rationalized in terms of redox potential differences. [Ni(SOD^{M1})] has a redox potential that is within the range necessary for SOD activity. Both **1** and **2** do not have an appropriate redox potential to act as an SOD. Bis-amine-ligated **1** has a Ni^{II}/Ni^{III} redox potential ($E_{1/2}$ > 1.2 V vs Ag/Ag$^+$)[20] far too positive to reduce superoxide, while bis-amide-ligated **2** will be incapable of oxidizing $O_2^{\bullet-}$ after accessing the Ni^{III} oxidation state ($E_{1/2}$ = −0.40 V vs Ag/Ag$^+$).[19] Therefore, both small-molecule NiN_2S_2 complexes are incapable of acting as SODs on the basis of redox potential arguments. Altogether, these reactivity results suggest that the Ni($S_2N^{amine}N^{amide}$) structural motif is electrochemically optimized to allow for Ni to display SOD activity.

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Supporting Information Available: Experimental details; analytical data for SOD^{M1} and [Ni(SOD^{M1})]; and SOD activity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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