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Nickel Superoxide Dismutase: Structural and Functional Roles of His1 and its H-bonding Network

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Abstract

Crystal structures of nickel-dependent superoxide dismutases (NiSODs) reveal the presence of a H-bonding network formed between the N-H of the apical imidazole ligand from His1 and the Glu17 carboxylate from a neighboring subunit in the hexameric enzyme. This interaction is supported by another intra-subunit H-bond between Glu17 and Arg47. In this study, four mutant NiSOD proteins were produced to experimentally evaluate the roles of this H-bonding network, and compare the results with prior predictions from DFT calculations. H1A-NiSOD, which lacks the apical ligand entirely, was crystallographically characterized and reveals that in the absence of the Glu17-His1 H-bond, the active site is disordered. Subsequent characterization using X-ray absorption spectroscopy (XAS) shows that Ni(II) is bound in the expected N₂S₂ planar coordination site. Despite these structural perturbations, the H1A-NiSOD variant is an active catalyst with 4% of WT-NiSOD activity. Three other mutations were designed to preserve the apical imidazole ligand, but perturb the H-bonding network: R47A-NiSOD, lacks the intra-molecular H-bonding interaction, E17R/R47A-NiSOD, which retains the intra-molecular H-bond, but lacks the inter-molecular Glu17-His1 H-bond, and E17A/R47A-NiSOD, which lacks both H-bonding interactions. These variants were characterized by a combination of techniques including XAS characterization of the nickel site structure, kinetic studies employing pulse-radiolytic production of superoxide, and EPR and chemical probes of the redox activity. The results indicate that in addition to the roles in redox tuning suggested by the computational models, the Glu17-His1 H-bond plays an important structural role in the formation of the Ni-hook motif that is a critical feature of the active site.

Keywords

superoxide dismutase (SOD); X-ray absorption spectroscopy (XAS); differential scanning calorimetry (DSC)

Superoxide dismutases (SODs) are metalloenzymes that protect aerobic organisms from oxidative stress caused by the superoxide anion radical ($O_2^{\bullet-}$) and its reaction products.⁽¹⁻⁴⁾ Elimination of superoxide is achieved by catalyzing the disproportionation reaction to produce molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) at rates approaching the diffusion limit. The catalysis proceeds by a “ping-pong” mechanism in which the redox-active metal cofactor cycles between oxidized and reduced states that differ by one electron (Equations 1-3).

Three classes of SODs exist based on amino acid sequence homology: CuZnSOD, FeSOD and MnSOD, and NiSOD. Like the Fe- and MnSODs, the more recently discovered NiSOD enzyme catalyzes the disproportionation of $O_2^{\bullet-}$ by cycling between M(III) and M(II) oxidation states.⁽⁵⁾ The CuZnSOD, FeSOD, and MnSOD proteins bind their respective catalytic metal cofactors using exclusively N/O-donor ligands (Figure 1).⁽⁶⁻⁸⁾ In contrast, NiSOD employs cysteine thiolate ligation to bring the Ni(III/II) couple into a the range relevant for SOD catalysis (~300 mV).⁽⁴⁾ In the reduced state of NiSOD, the Ni(II) cofactor adopts a four-coordinate planar geometry that features two thiolate ligands (Cys2 and Cys6), the N-terminal amine from His1, and a deprotonated amide from the Cys2 backbone.^(9, 10) In the oxidized state of NiSOD, the Ni(III) ion additionally binds the imidazole side chain of His1 via the N_δ donor atom, which results in a five-coordinate pyramidal geometry around the nickel ion with the His1 imidazole at the apex (Figure 1).^(9, 10)

The high-resolution crystal structures of WT-NiSOD from *S. seoulensis* and *S. coelicolor* also show that NiSOD forms a homohexamer comprised of four-helix bundle monomers that place the nickel sites in a roughly octahedral arrangement ~25 Å apart. The hexameric quaternary structure can be viewed as a dimer of trimers (Figure 2) where three monomer subunits are arranged like the legs of a tripod and two trimers interdigitate to form the hexamer. The hexamer is stabilized by hydrophobic, salt bridge, and H-bonding interactions. One important inter-subunit interaction involves a H-bonding network formed between the N-H of the apical imidazole ligand from His1 and the Glu17 carboxylate from a neighboring subunit. This interaction is further supported by an intra-subunit interaction between Glu17 and Arg47 (Figure 2). The crystal structures also reveal that the His1 side chain is disordered in the resting state of the enzyme, resulting in a hexamer that contains active sites

that are a mixture of either Ni(III)/His_{on} and Ni(II)/His_{off} forms, but where the His1-Glu17 H-bond is maintained in both structures.^(9, 10)

Density functional theory (DFT) computations have been used to investigate the role of the His1-Glu17-Arg47 H-bonding network in stabilizing the Ni(III) conformation of NiSOD, as well as its role in influencing the interaction between the His1 imidazole and the nickel cofactor.⁽¹¹⁾ Using the information from the crystal structures, five computational models were constructed and subjected to DFT energy minimizations: **ox¹⁻³**, **ox-His_{off}**, and **red**. In the **ox²** and **ox³** models of Ni(III)SOD, the H-bonding interactions between His1 and Glu17 (**ox²** and **ox³**), and additionally between Arg47 and Glu17 (**ox²**) were included. The results obtained indicated that the H-bonding interactions are important in adjusting the Ni-N₈His1 distance, and thus in tuning the energy of the redox active molecular orbital (d_z²) and thereby adjusting the redox potential of the Ni site.⁽¹¹⁾ In addition, the results obtained for the **red** model, which contains Ni(II) and lacks the axial imidazole ligand, and **ox-His_{off}**, which includes Ni(III) and the His1 imidazole in a non-bonding position that maintains the H-bond with Glu17 in the C₂-related subunit, indicated that the His1 imidazole side chain is also involved in stabilizing the Ni(III) state of NiSOD and in promoting metal-centered rather than ligand (thiolate) -centered redox chemistry.⁽¹¹⁾

In the present study, we have used an experimental approach employing site-directed mutagenesis to generate four *Streptomyces coelicolor* NiSOD mutant proteins to probe the roles His1-Glu17-Arg47 H-bonding network and test the predictions from the calculations. The four variants are: H1A-NiSOD, which lacks the apical imidazole and associated H-bond with Glu17; R47A-NiSOD, which retains the inter-subunit Glu17-His1 H-bond but lacks the Arg47-Glu17 interaction, E17R/R47E-NiSOD, which retains the intra-subunit Glu17-Arg47 H-bond, but lacks the inter-subunit interaction between Glu17 and His1; and E17A/R47A-NiSOD, which lacks all of these H-bonding interactions. The results of the studies are compared with those obtained for recombinant *S. coelicolor* WT-NiSOD and largely confirm the predictions from the calculations. All of the mutant proteins have impaired catalytic activity and are isolated primarily in the Ni(II) oxidation state. In addition, a crystal structure of the H1A-NiSOD variant shows a disordered active site, implying that the active site does not properly fold in the absence of the His1-Glu17 H-bonding interaction and demonstrating a critical structural role for the His1 – Glu17 H-bond. Collectively, our results indicate that both the presence of His1 and the His1-Glu17-Arg47 H-bonding network are critical features for the proper organization of the active site and for supporting redox catalysis.

Materials and methods

Site-Directed Mutagenesis

The H1A-NiSOD mutant protein was prepared by polymerase chain reaction (PCR) of the WT-*Streptomyces coelicolor* NiSOD gene (*sodN*) with a mutagenic 5'-primer and 3'-SODNU (supporting information Table SI-1). The PCR product was gel purified (1% agarose) and treated with T4 DNA polymerase to create overhangs complementary to the Ligation Independent Cloning (LIC) vector pET30 Xa/LIC (Novagen™). The insert was annealed to the vector and NovaBlue (Novagen™) competent cells were transformed with

the pET30 Xa/LIC plasmid containing the *sodN* mutant gene for selection. The plasmid containing mutagenic *sodN* was isolated using a plasmid mini prep kit (Qiagen) and DNA sequencing was performed to confirm the expected base sequence for the mutant (University of Massachusetts-Amherst DNA sequencing facility).

The R47A-NiSOD mutant protein was prepared by PCR of WT-NiSOD pET-30 Xa/LIC plasmid with mutagenic 5'- and 3'-primers and the E17A/R47A-NiSOD and E17R/R47E-NiSOD double mutations were generated by PCR of the R47A-NiSOD pET-30 Xa/LIC plasmid with mutagenic 5'- and 3'-primers using the QuikChange[®] XL Site-Directed Mutagenesis Kit (supporting information Table SI-1). NovaBlue (Novagen[™]) competent cells were transformed with the PCR product for mutant gene for selection. The plasmid containing mutagenic *sodN* was isolated using a plasmid mini prep kit (Qiagen) and DNA sequencing was performed to confirm the expected base sequence for the mutant (W. M. Keck DNA facility at Yale University, New Haven, CT). BL21 (DE3) pLysS (Novagen[™]) competent cells were transformed with the plasmid containing the desired mutation(s) and the variant protein was expressed and purified as previously described^(12, 13) and detailed below.

Expression, Purification, Processing, and Reconstitution of Mutant proteins

The H1A-NiSOD and R47A-NiSOD proteins were expressed and purified as previously reported.⁽¹²⁻¹⁴⁾ Single colonies were grown overnight at 37 °C with shaking in 10 mL Luria-Bertani broth, supplemented with chloramphenicol (cam) and kanamycin (kan) for selection. These cultures were added to 1L pre-warmed fresh media and grown to an OD₆₀₀ of 0.6 and then induced with 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3-5 hours. Cells were harvested by centrifugation, resuspended in 40 mL Ni-NTA binding buffer (10 mM imidazole, 50 mM sodium phosphate, 300 mM sodium chloride pH 8.0), and then frozen at -80 °C to lyse the cells. Low soluble protein expression was observed for E17A/R47A-NiSOD and E17R/R47E-NiSOD using the previously reported expression protocol, and inclusion bodies were observed in the cell pellet when a 37 °C induction temperature was used for expression. Therefore, these mutant proteins were expressed at a 28 °C induction temperature, and cells were harvested after 12-14 hours, and resuspended as described above. The cell harvests were thawed and treated with 100 μL of DNase I solution (10 mg/ml DNase I, 10 mM magnesium chloride, 20 mM Tris pH 7.5, 40% glycerol) at 37 °C until the viscosity of the solution was significantly reduced. The cell lysate was centrifuged for 5 min at 8,000 rpm and the supernatant was used for protein purification.

All chromatographic purifications employed an AKTA-FPLC (Amersham Biosciences). The cell lysate supernatant was loaded onto a column (Pharmacia HR10) containing Ni-NTA HisBind Superflow[™] resin (Novagen[™]) at 3 ml/min with Ni-NTA binding buffer. Once the absorbance at 280 nm returned to the baseline value, the buffer was changed to 12% elute buffer (250 mM imidazole, 50 mM Tris, 300 mM sodium chloride pH 8.0) in one step, and the column was washed with 7 column volumes of 12% elute buffer. The fusion protein was then eluted from the column using 70% elute buffer. Electrospray ionization mass

spectroscopy (ESI-MS) was used to confirm the molecular weight (MW) of the expected fusion protein (*vide infra*).

To remove imidazole from the protein sample, the fusion protein was loaded onto a column (Pharmacia HR10) containing Q-Sepharose resin at 3 mL/min with bind buffer (50 mM Tris pH 8.50). Once the absorbance at 280 nm returned to the baseline value, the buffer was changed to 40% elute buffer (50 mM Tris, 500 mM sodium chloride pH 8.5) in one step, and the fusion protein was then eluted from the column and collected. The eluted sample was adjusted to factor Xa buffer conditions (5 mM CaCl_2 , 50mM Tris, 100mM NaCl, pH 8.0) for cleavage of the N-terminal fusion peptide with factor Xa (EMD). The concentration of fusion protein in factor Xa cleavage buffer was determined using a bicinchoninic acid (BCA) assay. The assay was performed using the Enhanced Test Tube Protocol outlined in Pierce's BCA™ Protein Assay Kit instruction manual. Factor Xa was then added (30 μL 2.1 U/ μL factor Xa/50 mg fusion protein) and the mixture was incubated at 4 °C. The extent of cleavage was monitored using 16% SDS-PAGE. Processed protein was reduced with a five-fold excess dithiothreitol (DTT) and reconstituted with a three-fold excess of NiCl_2 in an anaerobic glove box (Coy Laboratory Products, Inc.). Excess DTT and Ni were removed by the same FPLC purification protocol utilized to remove imidazole in the procedure described above.

A nickel-loaded sample of H1A-NiSOD was incubated with a 15-fold excess of imidazole to produce H1A_{IMD}-NiSOD.

ESI-MS Analysis

To determine the MW of the NiSOD expression products, ESI-MS analysis was performed using a Bruker Esquire mass spectrometer equipped with an HP-HPLC. Analysis was performed under protein denaturing conditions, 0.1% formic acid and skimmer voltage set to 120 V, and samples were prepared to a final concentration of 20 μM monomer (Table 1).

Metal Analysis

A PerkinElmer Optima 4300 DV inductively coupled plasma-optical emission spectrometer (ICP-OES) was used to quantify the nickel content of the reconstituted mutant proteins. This instrument is equipped with a 40 MHz free-running generator and a segmented-array charge-coupled device detector. The sample introduction system consisted of a concentric nebulizer with a cyclonic spray chamber. The concentration of nickel in each sample was determined at $\lambda = 231.604$ nm.

Quaternary Structure and Stability

To determine the oligomeric state of the proteins, size-exclusion chromatography was performed using a Superdex 75 15/300 GL (GE Lifesciences) column. The column was standardized with albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa). A standard curve was constructed by plotting V_e/V_0 vs MW (where V_e = elution volume of the peak, and V_0 = elution volume of blue dextran), and the data were fit with a second order polynomial. The apparent MW determined (Table 2) is in agreement with calculated MW values based on the elution volume. Since NiSOD is a

tightly organized hexamer of four helix bundles, the protein is eluted at lower elution volumes than typical for ~80 kDa proteins. The corrected MW corresponds to adjusted molecular values based on the known MW and elution volume of WT-NiSOD. WT-NiSOD and NiSOD variants were injected onto the column at a concentration of 480 μ M (monomer) in 20 mM Tris, 100 mM NaCl pH 8.0, and the retention volume of the peak(s) (V_e/V_0) from each chromatogram was analyzed using the standard curve to determine the molecular weights of the eluted proteins (see Figure 4 inset).

Melting temperatures (T_m) were measured with a Microcal VP-DSC with a 0.5 mL sample and reference cells. Samples were concentrated to 100-140 μ M monomer in 50 mM Tris buffer. Protein samples and blank 50 mM Tris buffer were degassed under vacuum for 10 min and syringed into the cells using a pulsing motion to expel air bubbles. Samples were run at 30 psi over 25-100 $^{\circ}$ C at a scan rate of 30 $^{\circ}$ C/hour. Baseline correction and normalization were performed with the Microcal interface to the Origin software package, and T_m (s) were taken to be the peak maxima of the thermogram.

Crystallography of H1A-NiSOD

Crystals of H1A-NiSOD were grown by hanging drop vapor diffusion as previously described.⁽¹³⁾ Prior to cryo-cooling and data collection, the crystals were transferred into growth buffer supplemented with 40% glycerol. X-ray diffraction data (266 frames, 0.75 degrees per frame) were collected at APS beamline X6A at a wavelength of 0.9184 $\text{\AA}$. Data were integrated and merged in HKL2000⁽¹⁵⁾ to a resolution of 2.01 $\text{\AA}$. The structure was solved by Fourier synthesis using PDB file 3G4X. The structure was refined in the CCP4 program Refmac⁽¹⁶⁾ and built using Coot.⁽¹⁷⁾

To determine the nickel content of the crystals, a sample of the H1A-NiSOD crystals was dissolved in purified water and the protein concentration was determined using the absorbance at 280 nm. The sample was then diluted to a calculated concentration of 50 ppb based on nickel content determined by ICP-OES. Solution standards of nickel were made in purified water at a concentration of 1-500 ppb and the H1A-NiSOD sample and nickel standards were analyzed by inductively-coupled plasma mass spectrometry (ICP-MS) in triplicate.⁽¹⁸⁾ The area under each peak was calculated and a standard curve was constructed to back calculate the [Ni] in the protein sample.

X-Ray Absorption Spectroscopy

X-ray absorption spectroscopy (XAS) data collection and analysis were performed as previously described using a model that includes multiple-scattering from second-coordination sphere atoms in the chelate rings formed as a result of the bidentate coordination of His1 (N-terminal amine and imidazole side chain) and Cys2 (amidate and thiolate sidechains).^(13, 14, 19) Nickel *K*-edge XAS data were collected on beamline X3B (and X9B) at the National Synchrotron Light Source (Brookhaven National Laboratory). Samples of frozen protein solutions (1-3 mM, based on nickel content, in 20 mM Tris•HCl, pH 8.0) were placed in polycarbonate holders inserted into aluminum blocks and held near 50 K using a He displax cryostat. The ring conditions for data collection were 2.8 GeV and 120-300 mA. A sagittally focusing Si(111) double crystal monochromator and a 13-channel

Ge fluorescence detector (Canberra) were used for data collection. X-ray absorption near-edge spectroscopy (XANES) data were collected from ± 200 eV relative to the nickel *K*-edge. The edge energy reported was taken to be the maximum of the first-derivative of the XANES spectrum. Extended X-ray absorption fine structure (EXAFS) data were collected to 9307 eV ($k = 16 \text{ \AA}^{-1}$). The X-ray energy for the *K*-edge of nickel was internally calibrated to 8331.6 eV using transmission data from a nickel foil. The data shown are the average of 5-6 scans and were analyzed using the EXAFS123 software package for XANES data and the SixPack software package for EXAFS data.⁽²⁰⁾ Scattering parameters for SixPack fitting were generated using the FEFF 8 software package.⁽²¹⁾ The SixPack fitting software builds on the ifeffit program.⁽²²⁾ The coordinates of the oxidized and reduced NiSOD models obtained from density functional theory geometry optimizations were used as input files for FEFF 8.⁽¹¹⁾ Two sets of input files were used; one that included all non-hydrogen atoms from the **ox**¹ computational model, and another that eliminated the apical imidazole ligand, as in the **red** model.⁽¹¹⁾ Comparison of the fits obtained using these two models were used to assess ligation by the His1 imidazole. To compare different models to the same data set, ifeffit uses three goodness of fit parameters; namely, χ^2 (Equation 4), reduced χ^2 , and the *R*-factor (Equation 5), where *N*_{idp} is the number of independent data points, *N* ϵ^2 is the number of uncertainties to minimize, *Re*(*f*_{*i*}) is the real part of the EXAFS fitting function, and *Im*(*f*_{*i*}) is the imaginary part of the EXAFS fitting function. Reduced $\chi^2 = \chi^2 / (N_{\text{idp}} - N_{\text{vars}})$ (where *N*_{vars} is the number of refining parameters) and represents the degrees of freedom in the fit.

$$\chi^2 = \frac{N_{\text{idp}}}{N_{\epsilon^2}} \sum_{i=1}^N \left\{ [Re(f_i)]^2 + [Im(f_i)]^2 \right\} \quad (4)$$

Iffeffit also calculates the *R*-factor for the fit, which is given by Equation 5 and is scaled to the magnitude of the data, making it proportional to χ^2 . To compare different models (fits), the *R*-factor and reduced χ^2 parameters can be evaluated to determine which model provides the best fit, in which case both parameters should be minimized. Although the *R*-factor will always improve with an increasing number of adjustable parameters, reduced χ^2 will go through a minimum and then increase, indicating that the model is over fitting the data.

$$R = \frac{\sum_{i=1}^N \left\{ [Re(f_i)]^2 + [Im(f_i)]^2 \right\}}{\sum_{i=1}^N \left\{ [Re(\tilde{\chi}data_i)]^2 + [Im(\tilde{\chi}data_i)]^2 \right\}} \quad (5)$$

EPR and Spin Quantification of as-isolated, dithionite reduced, and IrCl₆ oxidized NiSOD mutants

X-band EPR spectra were obtained on a Bruker ELEXSYS E-500 X-band spectrometer. The data were collected over a 1500 Gauss field centered at 3100 Gauss. The modulation receiver and signal channel parameters were a modulation frequency of 100 kHz, a modulation amplitude of 10 Gauss, and a time constant of 327 ms. The microwave power

was set to 20 mW with a frequency of 9.58 GHz. To determine the number of Ni(III) centers present in each NiSOD variant, spin integration was performed on each EPR spectrum. The resulting EPR spectra were integrated twice and the concentration of Ni in each sample was determined using ICP-OES ($\lambda=231.604$ nm). Reduced samples were first treated with 10 molar equivalents of sodium dithionite in an anaerobic environment and analyzed by EPR for the presence(absence) of Ni(III). The reduced samples were buffer exchanged anaerobically with fresh 20 mM Tris, pH 8.5 to remove excess reducing agent and treated with two molar equivalents of IrCl_6 to produce the oxidized samples. The oxidized samples were then analyzed by EPR spectroscopy for Ni(III) content.

Kinetics

Kinetics experiments used pulse radiolytic generation of $\text{O}_2^{\bullet-}$ and were carried out using a 2 MeV Van de Graaff accelerator at Brookhaven National Laboratory, as previously described.^(13, 14) Superoxide radicals were generated upon pulse radiolysis of an aqueous, air/ O_2 saturated solution containing 10 mM phosphate, 30 mM formate, and 5 μM ethylenediaminetetraacetic acid (EDTA). Catalytic rate constants were obtained by monitoring the disappearance of $\text{O}_2^{\bullet-}$ at 260 nm in the presence of micromolar concentrations of mutant NiSOD. The path length of the quartz cell used was 2.0 cm and a 100-700 ns pulse width was chosen, resulting in the generation of 1-7 μM $\text{O}_2^{\bullet-}$ per pulse. The reported rate constants are based on metal concentration, with the assumption that all nickel ions are specifically bound and equally contributing to $\text{O}_2^{\bullet-}$ dismutation.

The transient intermediates of WT- and H1A-NiSOD were characterized by recording the absorption as a function of wavelength during pulses from the Van de Graaff accelerator. The extinction coefficients were calculated using a simple bimolecular mechanism for the formation of the absorbance ($\text{O}_2^- + \text{H1A-Ni(II)SOD}$) and the intermediate extinction coefficient was calculated assuming a first-order process for the disappearance of the absorbing species. Data was collected at 80 μM H1A-NiSOD.

Results

Protein Characterization

All of the expressed fusion proteins had the MWs expected by ESI-MS for the intended single and double amino acid substitutions (Table 1). Upon reconstitution of proteins processed to remove the N-terminal extension, each of the mutant proteins bound nickel at varying stoichiometric ratios that were $< 1:1$ using the same method for removing excess and non-specifically bound Ni(II) (*vide supra*) (Table 1). For WT-NiSOD and E17A/R47A-NiSOD, the Ni:protein ratio was ~ 0.9 , close to 1:1. The other mutants had lower retention of the Ni(II) ion, ranging from 0.6 for R47A-NiSOD to 0.19 for H1A-NiSOD. Unlike the native enzyme, which is golden-brown in color due to $\text{S(Cys)} \rightarrow \text{Ni(III)}$ charge transfer (LMCT) transitions near 380 nm, the H1A-NiSOD and E17A/R47A-NiSOD mutants were a lighter tan color. This difference likely results from the lower nickel loading and the fact that all or most of the nickel centers are in the reduced, Ni(II), state in these variants (*vide infra*). Size-exclusion chromatography (SEC) of the reconstituted NiSOD variants gave rise to an elution peak with a retention volume corresponding to the molecular weights appropriate for

homohexamers, as is the case for WT-NiSOD (Table 2 and Figure 4). In addition, the H-bonding mutants, R47A-, E17R/R47A- and E17A/R47A-NiSOD showed small peaks with estimated MW close to those calculated for trimers, suggesting that perturbation of the H-bonding network weakens the ‘dimer of trimers’ hexameric structure.

The overall protein thermal stability was altered by the amino acid substitutions as revealed by the DSC data. Specifically, the variants generally have 10 – 20 °C lower melting temperatures (T_m) (Table 1 and Figure 3), and less stable homohexamers compared to WT-NiSOD. The DSC thermogram of WT-NiSOD exhibits an asymmetric curve, suggesting a complex thermal denaturation of the protein (Figure 3).^(13, 14) A likely interpretation is that the lower temperature tail represents dissociations of the hexamer, while the sharp transition represents unfolding of the helical subunits. This hypothesis is supported by the fact that WT-NiSOD and the R47A-NiSOD variant, which contain an inter-subunit His1-Glu17 H-bond, show pronounced tailing, whereas the variants lacking this H-bond (or the apical His imidazole altogether) exhibit a more symmetrical peak, suggesting a more easily dissociated hexamer results from loss of the inter-subunit H-bond. This interpretation is also consistent with the SEC data (*vide supra*).

Electron Paramagnetic Resonance

Amino acid substitutions that alter the H-bonding network present in WT-NiSOD affect the oxidation state/redox potential of the nickel site, as revealed by EPR spectra of the variant proteins. Resting (as-isolated) native and recombinant WT-NiSOD exhibit a rhombic EPR spectrum that arises from a five-coordinate, low-spin ($S = 1/2$) Ni(III) center, with the unpaired electron residing in the Ni d_{z^2} -based molecular orbital (MO). In addition, the spectrum reveals N-hyperfine structure on the high-field component that arises from coordination of the apical His1 imidazole ligand. Spin quantitation indicates that the EPR signal intensity only accounts for half of the nickel atoms in the sample; therefore, as-isolated WT-NiSOD is approximately half oxidized and half reduced.⁽¹³⁾ As-isolated R47A-NiSOD and E17R/R47E-NiSOD display EPR signals with lower intensities that are very similar to the WT-NiSOD Ni(III) signals (Figure 5), including the N-hyperfine structure, indicating that the Ni(III) species retain the His-on structure. As-isolated H1A-NiSOD and E17A/R47A-NiSOD, as well as reduced samples of R47A-NiSOD and E17R/R47E-NiSOD_{red} lack this EPR spectrum; all are EPR-silent (data not shown), indicating that only Ni(II) is present. Double integration of the R47A-NiSOD and E17R/R47E-NiSOD EPR signals and comparison with the Ni content of the samples (ICP-OES) indicate that the samples contain ~15% and 8% Ni(III), respectively. This result shows that a perturbation of the active site H-bonding network affects the redox potential of the Ni site and/or the stability of the WT-like oxidized Ni(III) active site structure. Oxidation of as-isolated R47A-, E17A/R47A-, and E17R/R47E-NiSOD with IrCl_6 did not result in an increase in EPR signal intensity.

MCD

VT VH-MCD spectra were obtained as previously described.⁽¹¹⁾ Owing to low sample concentration, the signal to noise ratio of the spectra obtained is not optimal, but the data are sufficient to establish that the paramagnetic Ni(III) fraction varies from variant to variant

(consistent with the EPR results presented here) and that the Ni(II) fraction is low-spin and diamagnetic, as in WT-NiSOD (see supporting information).

Crystal structure of H1A-NiSOD

The X-ray crystallographic structure of H1A-NiSOD shows that the N-terminal region from residues 1 to 7 is disordered, despite the spectroscopic observations that the metal is bound in the active site. Overall, the H1A-NiSOD structure resembles that of apo-WT-NiSOD where the N-terminus is unresolved. The H1A-NiSOD crystals contained a nickel to protein ratio of 0.13:1, in close agreement with samples prepared as solutions for DSC, EPR, and XAS analysis. Collectively, these results suggest that while nickel binds to the N-terminal region of H1A-NiSOD, the metal binding region is highly mobile compared to that of nickel-bound WT-NiSOD.

X-ray Absorption Spectroscopy

X-ray absorption spectroscopy provides local structural information regarding the nickel sites. As such, structural information regarding the fraction of nickel-bound variants could be obtained even in cases where the portion of the protein comprising the “Ni hook” motif was disordered in the crystal structures. However, such information represents an average of all of the nickel site structures in the sample. Nonetheless, XAS data of as isolated WT-NiSOD (a 50:50 mixture of Ni(III) and Ni(II) centers) clearly contain evidence for the coordination of the apical His1 imidazole that is lacking in the reduced sample, where this ligand is not bound (Table 3 and Figure SI-2).⁽²³⁾

XANES

The XANES region of the nickel *K*-edge XAS spectrum provides information about the nickel center coordination number and geometry and is the basis for the geometry determination shown in Table 4. The spectrum obtained for H1A-NiSOD has a peak near 8331 eV that is associated with a Ni 1s → 3d electronic transition (Table 4), the area of which is consistent with a planar four- or six-coordinate (centrosymmetric) nickel site.⁽²⁴⁾ The presence of a peak near 8336 eV associated with a Ni 1s → 4p_z electronic transition in the H1A-NiSOD spectrum is consistent with a planar four-coordinate geometry.⁽²⁴⁾

An attempt to recover the WT-NiSOD structure by adding imidazole to the buffer did not produce the expected five-coordinate pyramidal imidazole adduct. Instead, the XANES spectrum obtained for H1A_{IMD}-NiSOD is consistent with a six-coordinate Ni site. The spectrum features a peak near 8331 associated with a Ni 1s → 3d electronic transition (Table 4), the area of which is consistent with a centrosymmetric arrangement of ligands (*i.e.*, a four- or six-coordinate nickel site), and the absence of a peak or shoulder near 8336 eV is consistent with a six-coordinate geometry.⁽²⁴⁾ Compared with WT-NiSOD, the XANES data for the H1A_{IMD}-NiSOD mutant also feature an increase in the white line region (0-50 eV above the edge) that is indicative of an increase in the ratio of N/O-donors to S-donors,⁽²⁴⁾ demonstrating that the addition of imidazole promotes a coordination change at the active site.^(12, 13, 23)

The XANES spectra obtained for the as-isolated H-bonding variants R47A-, E17R/R47E-, and E17A/R47A-NiSOD have peaks near 8331 eV that are associated with a Ni 1s $\rightarrow$ 3d electronic transition, the intensities of which are consistent with the presence of a five-coordinate nickel site (Table 4).⁽²⁴⁾ Moreover, the presence of shoulders near 8336 eV in the R47A-, E17A/R47A-, and E17R/R47E-NiSOD spectra, which are associated with a Ni 1s $\rightarrow$ 4p_z electronic transition, are consistent with a pyramidal five-coordinate geometry.⁽²⁴⁾

EXAFS

The EXAFS region of the XAS spectrum provides information regarding the metal ligands, including the donor-atom types, numbers, and bond distances. Analysis of the EXAFS features arising from atoms in the first coordination sphere (single-scattering analysis) indicates that the best fits for the H1A-NiSOD and reduced H-bonding variants feature an N₂S₂ ligand donor atom set that is consistent with the XANES results indicating a planar four-coordinate nickel site (Tables SI-2, SI-5, SI-7, and SI-9). Single-scattering analysis of EXAFS data obtained for the as-isolated R47A-, E17A/R47A-, and E17R/R47E-NiSOD variants indicate that the Ni sites associated with these proteins feature N₃S₂ ligand donor atom sets, which is consistent with the XANES results indicating a pyramidal five-coordinate nickel site (Tables SI-4, SI-6, and SI-8). Since the NiSOD sequence contains only three S-donor ligands (Cys2, Cys6, and Met28), and mutation of Met28 has no effect on the NiSOD active site properties, along with the fact that signatures characteristic of S-donor ligands are present in the EXAFS spectra of all the NiSOD variants, the EXAFS spectra indicate that the nickel cofactor is binding in the same Ni-hook domain involving both Cys residues, as in WT-NiSOD.

Multiple-scattering analysis that includes data from scattering atoms in the second and third coordination spheres of the nickel center supports the presence of one imidazole ligand in the as-isolated H-bonding variants, consistent with nickel binding to the His1, imidazole and shows no imidazole ligand for H1A-NiSOD, consistent with the loss of the apical His1 ligand. Table 4, Figure 7, and Figure 9 compare the best fits obtained for all NiSOD variants with and without paths derived from imidazole binding (second coordination sphere C and third coordination sphere N and C atoms). These fits also include the main chain carbon atoms from His1 and Cys2, which are involved in five-member chelate rings, and are thus held at specific distances that are similar to those involving the second coordination sphere imidazole C atoms. The best fits for the Ni sites in the as-isolated mutant proteins were invariably those that included an imidazole ligand.

Kinetics

The rates of superoxide dismutation by the variants with altered H-bonding interactions in the active site were determined by monitoring the disappearance of the optical absorption feature of pulse-radiolytically generated superoxide at 260 nm, in the presence and absence of the NiSOD mutant proteins. The catalytic rate observed for WT-NiSOD at pH 7.5 is $0.71 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$,⁽¹³⁾ as determined by the first-order disappearance of superoxide, is proportional to the NiSOD concentration, and displays a pH-independent catalytic rate over the range of pH $\sim$ 6-10, indicating that the protons required to produce H₂O₂ are derived from the protein rather than bulk solvent (Figure 10). In contrast, the mechanism for

spontaneous superoxide disproportionation is second order in $[O_2^-]$, is highly pH-dependent, and proceeds at a rate of $\sim 2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ near neutral pH and at the $[O_2^-]$ used here.^(23, 25) The disappearance of superoxide in the presence of all the variants displayed a rate that was proportional to enzyme concentration and pH-independent, indicating that these variants are catalysts, and catalyze superoxide dismutation according to a common mechanism (Table 1 and Figure 10). In the case of H1A- and E17A/R47A-NiSOD (variants lacking a His1-Glu17 H-bond) the catalytic rate of superoxide dismutation is reduced by a factor of >10 over WT-NiSOD, and the presence of imidazole in the H1A_{IMD}-NiSOD sample did not recover activity. For R47A- and E17R/R47E-NiSOD, the catalytic rate of superoxide dismutation is reduced by a factor of $\sim$ five (Table 1 and Figure 10). This level of activity is between that observed for WT-NiSOD and H1A-NiSOD, and indicates that variants that retain a possible His1-Glu17 H-bond are more active than those that prevent this interaction, but are still impeded by the structural effects of the amino acid substitutions.

Spectral data taken during the single turnover of as-isolated H1A-NiSOD with O_2^- show evidence for the formation of species that are not observed for WT-NiSOD. The spectrum obtained from reduced WT-NiSOD shows a rapid rise in absorbance near 380 nm that persists and has been assigned to $S \rightarrow \text{Ni(III) LMCT}$,⁽¹¹⁾ consistent with the formation of stable Ni(III) sites. In contrast, the spectrum obtained for as-isolated H1A-NiSOD (reduced) shows a rapid rise in absorbance near 390 nm, followed by a slow decay (much slower than catalysis) that is independent of the $[O_2^-]$ and occurs in two steps ($k = 40 \text{ s}^{-1}$ and $= 3 \text{ s}^{-1}$), and thus involves a process and a transient species that are not observed for WT-NiSOD (Figure 11). The data are consistent with the formation of a distinct Ni(III) species, and its subsequent loss by a process that is independent of $[O_2^-]$ suggests an internal redox process. The nature of the observed species awaits further spectroscopic investigations.

Discussion

High-resolution crystal structures of NiSOD reveal the presence of a H-bonding network formed between the N-H of the apical imidazole ligand from His1 and the Glu17 carboxylate from a neighboring subunit related by a two-fold axis of rotation (Figure 2).^(9, 10) This interaction is supported by another intra-subunit H-bond between Glu17 and Arg47. In this study, four mutant NiSOD proteins were produced to experimentally evaluate the roles of the inter-subunit H-bonding network involving the apical His1 imidazole ligand and Glu17 and Arg47 in a neighboring subunit, and to test the predictions based on the DFT calculations.⁽¹¹⁾ H1A-NiSOD lacks the apical imidazole ligand and is thus relevant to the **red** and **ox-His_{off}** models. E17A/R47A-NiSOD and E17R/R47E-NiSOD remove all the H-bonds or just the Glu17 – His1 H-bond, respectively, and may be compared with the **ox¹** model, which lacks the Glu17 – His1 H-bond. R47A-NiSOD removes the Arg47 – Glu17 intra-subunit H-bond, while retaining the Glu17 – His1 inter-subunit H-bond, and is comparable to the **ox³** model. The **ox²** model represents WT-NiSOD with respect to the H-bonding network.

The **ox¹** and **red** models reproduced the equatorial ligand distances for the nickel sites within 0.05 Å when compared to the crystallographic distances.⁽¹¹⁾ However, a large discrepancy was noted between the DFT-optimized models and the crystallographic Ni-

$N_{\text{imidazole}}$ bond lengths. In the ox^1 model, the optimized axial N-donor distance was 2.07 Å, much shorter than the distance observed in the crystal structures (~2.3 Å). Analysis of the EXAFS spectra for Ni-L distances for low-spin Ni(II) sites reveals that the basal ligands contribute short Ni-N and Ni-S distances at ~1.9 Å and ~2.2 Å, respectively, as seen for dithionite-reduced WT-NiSOD (Tables 3). Using the protocol described above, and comparing fits with and without multiple-scattering from an imidazole ligand, the $N_{\text{imidazole}}$ distance in WT-NiSOD was determined to be 2.09(1) Å (Table 3), in excellent agreement with the geometry-optimized calculation.

In addition, the computational studies concluded that the His1 imidazole ligand was important to the electronic structure of the Ni(III) site by promoting nickel-centered rather than S-centered redox chemistry, and that the Ni-imidazole distance was influenced by the H-bonding network in a way that was important to tuning the energy of the redox active $Ni\ d_z^2$ orbital, and thus is an important adjustment on the redox potential of the site.

H1A-NiSOD

In addition to preventing imidazole coordination to the oxidized Ni(III) site, the loss of the apical imidazole had a dramatic effect on the structure of the “Ni-hook” motif. In the crystal structure of H1A-NiSOD, the N-terminal residues prior to Val8 are disordered, suggesting that the H-bond between His1 and Glu17 plays a critical role in defining the structure of the site, since all the ligands required to support coordination of the Ni(II) site structure are present. Analysis of the XAS spectrum of H1A-NiSOD confirms low-spin Ni(II) binding to the expected planar four-coordinate N_2S_2 ligand environment (EXAFS: 2 Ni-N = 1.91(3) Å; 2 Ni-S = 2.18(1) Å, Table 4), and the Ni(II) site is structurally very similar to the planar site in dithionite-reduced WT-NiSOD found using the protocol described here (2 Ni-N = 1.87(3) Å; 2 Ni-S = 2.15(1) Å, Table 3) or previously (2 Ni-N = 1.91(1) Å; 2 Ni-S = 2.16(1) Å).⁽²³⁾

Prior crystallographic results show that the Glu17 – His1 H-bond is maintained in the structures of both oxidized (Ni(III)) and reduced (Ni(II)) NiSOD,^(9, 10) but loss of the H-bond in H1A-NiSOD results in similar disorder to that observed for the apoenzyme, where it was inferred that the structure of the “Ni-hook” depends on proper coordination of the metal cofactor. The loss of the “Ni-hook” structure observed for H1A-NiSOD implies that the His1-Glu17 H-bond plays an important role in stabilizing the structure of this motif, and is consistent with the failure of chemical rescue of this variant. The XAS data presented show that the addition of imidazole to solutions of H1A-NiSOD produces a six-coordinate complex that features either one or two imidazole ligands and does not lead to either the WT-NiSOD “Ni-hook” structure, or to increased catalytic activity (*vide infra*). The WT-NiSOD EPR signal is also not recovered in H1A_{IMD}-NiSOD, and the active site remains in the Ni(II) oxidation state. Equilibria between planar four-coordinate complexes and six-coordinate complexes are common in Ni(II) coordination chemistry:

Often, a five-coordinate intermediate is not observed because the $5 \rightleftharpoons 6$ equilibrium lies further to the right than the $4 \rightleftharpoons 5$ equilibrium. Thus, one apparent function of the His1 – Glu17 H-bond is to fold the Ni-hook in a way that prevents the formation of a six-coordinate Ni site. Although the nickel site is near the surface of the WT-NiSOD, it is effectively protected from solvent access by the hook motif. In the properly configured “Ni-hook,” Val8 blocks access to the sixth coordination position, opposite the imidazole side-chain of His1. This would not be the case in the more mobile “Ni-hook” observed for H1A-NiSOD.

The $4 \rightleftharpoons 6$ equilibrium would also alter the electronic properties of the active site, as it would involve conversion from a low-spin Ni(II) ion to a high-spin Ni(II) ion since six-coordinate Ni(II) centers are invariably high-spin.⁽²⁶⁾ The EXAFS analysis shows elongated M-L distances that support this, in particular a Ni-S distance of 2.28(3) Å (~0.1 Å longer than the low-spin distance, Table 4). Such high-spin Ni(II) centers have also been observed in Cys-to-Ser mutants of NiSOD, which are catalytically inactive.^(14, 27) The formation of a high-spin six-coordinate complex for the disordered “Ni-hook” domain may also sterically prevent folding into the “Ni-hook” structure that features a His1 imidazole-Glu17 H-bond, a possible explanation for why exogenous imidazole does not complement the loss of the His1 imidazole ligand.

Analysis of the kinetics data reveals that while H1A-NiSOD is only ~4% as active as WT-NiSOD at pH = 7.5, it is still a catalyst, implying that a one-electron oxidized state is still accessible. The computed electronic structure for the **ox-His_{off}** model indicated that the oxidation product is more ligand-centered without the apical imidazole ligand,⁽¹¹⁾ and offers a possible explanation for the instability of the oxidation product of H1A-NiSOD observed by spectroscopy. The Ni(III) species formed by oxidation of H1A-NiSOD with O₂[−] is unstable, and its decay could represent reduction of the Ni(III) center by the thiolate ligands, a scenario that is also consistent with the lack of a kinetic dependence on the [O₂[−]].

H-bonding variants

Three additional NiSOD variants were designed to preserve the apical imidazole ligand, but perturb the H-bonding network in various ways. Qualitative analyses of the Ni K-edge EXAFS spectra from a number of NiSOD samples have shown that binding the His1 side chain results in two resolved features in the Fourier-transform (FT)-EXAFS spectrum that arise from the first-coordination sphere ligands, whereas in the absence of the apical ligand, only one broad feature is observed.⁽¹⁴⁾ This presumably arises from the presence of a unique longer Ni-N vector in samples with an apical imidazole ligand. This observation is true of oxidized vs. reduced WT-NiSOD, and for H1A-NiSOD, which has only one broad feature in the FT-EXAFS spectrum. Using this criterion, all of the mutant proteins featuring a disrupted H-bonding network, but retaining the imidazole ligand, have the apical imidazole bound in the as-isolated samples.

For R47A-NiSOD, the amino acid substitution removes the intra-subunit interaction between Glu17 and Arg47, but preserves the His1-Glu17 H-bond. According to the **ox³** computational model, this is expected to increase the strength of the Glu17 – His1 H-bond relative to WT-NiSOD, and result in a longer Ni-Nimidazole distance relative to WT-NiSOD. The distance found by EXAFS analysis (2.19(7) Å, Table 4), is in agreement with

the prediction from the ox^3 computational model (2.14 Å).⁽¹¹⁾ In the remaining two mutants, E17A/R47A- and E17R/R47E-NiSOD, the Glu17 – His1 H-bond is absent, and a short Ni-Nimidazole bond is predicted by the ox^1 model.⁽¹¹⁾ Although the FT-EXAFS spectra of both variants show the two peaks expected for coordination of the His1 imidazole group, and while the best EXAFS fits obtained include the imidazole ligand, the improvement of the fits is not sufficient to make a clear case for the presence or absence of this ligand. It may be that there is a more significant fraction adopting the His-off structure in these samples relative to either WT-NiSOD or R47A-NiSOD. Nonetheless, the Ni-Nimidazole distances obtained from EXAFS fits that include the imidazole ligand are shorter (1.97(5) Å and 2.12(3) Å, respectively, Table 4) than for R47A-NiSOD.

In addition to the structural effects observed for the H-bonding mutants, the samples show perturbed catalytic activity that correlates with the intensity of the EPR spectrum obtained. The sample that retains a Glu17 - His1 H-bond, R47A-NiSOD, has a five-fold reduction in activity and ~15% of the Ni in the as-isolated sample is Ni(III), compared with 50% for WT-NiSOD.⁽¹³⁾ For E17R/R47E-NiSOD, the rate is reduced by a factor of ~six and EPR indicates ~8% Ni(III), and for E17A/R47A-NiSOD it is reduced by a factor of >ten and the sample is EPR silent, as is the case for H1A-NiSOD. The correlation suggests that the variants have impaired ability to access the oxidized, Ni(III) state for catalysis. The order also reflects the severity of the disruption to the H-bonding network: R47A- < E17R/R47E- < E17A/R47A-, H1A-NiSOD, and points to the importance of the H-bonding network in the redox chemistry of the active site.

One possible explanation for the trend in activity of the variant NiSODs is the one suggested by computational chemistry: that changes in the Glu17-His1 H-bond affect the interaction between the apical imidazole ligand and the Ni, resulting in a perturbation of the redox-active d_z^2 orbital, and perturbing the redox chemistry. This is consistent with studies of small polypeptides that model the "Ni-hook" motif.⁽²⁸⁻³⁰⁾ However, the models show a large H/D kinetic isotope effect⁽³¹⁾ that is not observed in the wild-type enzymes, where the reaction rate is diffusion limited, and indicate that the models catalyze superoxide disproportionation by a distinct mechanism. Nonetheless, the removal of the His imidazole ligand in the models affects the redox potential and activity in a way that is analogous to the H1A mutation; the complexes are isolated as Ni(II) species with planar N_2S_2 coordination, with increased redox potentials for the Ni(II/III) couple, and reduced catalytic activity (over an order of magnitude lower).⁽²⁸⁾ Further, studies of model systems where the imidazole ligand is modified, such as with N-Me, N-DNP, and N-Tos, show that the Lewis basicity of the ligand correlates with activity,^(29, 30) supporting the hypothesis that subtle electronic changes due to the H-bonding network involving the His1 donor are directly linked to optimizing the redox chemistry in the enzyme. However, loss of the Glu17-His H-bond should result in a stronger Ni-Nimidazole interaction, a destabilized d_z^2 orbital, and a lower redox potential that favors Ni(III), which is counter to impaired ability to access Ni(III) that is most consistent with the data. An alternative interpretation is a two-state model wherein the Ni(III) species represents a fraction of the protein that has a properly folded "Ni-hook" motif, and therefore a redox-active nickel site, and that the redox inactive fraction represents protein molecules with an unfolded "Ni-hook". This is more consistent with the data, since

weakening the Glu17-His1 H-bond should favor a higher fraction in the unfolded and redox-inactive state. Further, the fact that oxidations with NaIrCl₆ failed to increase the intensity of the Ni(III) signals in the H-bonding mutants suggests that the low intensity of the EPR signal is not due solely to a minor perturbation of the redox potential and is more consistent with the “all-or-nothing” character of the two-state model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Supporting information available: Ten tables summarizing mutagenic primers used for mutant preparations, EXAFS fits used in analyzing the EXAFS data, and MS-ICP nickel standards and calculated Ni content for H1A crystals. Six figures illustrating the XANES of dithionite-reduced native NiSOD and recombinant WT-NiSOD, EXAFS analysis of dithionite-reduced native NiSOD and recombinant WT-NiSOD, the standard curve of MS-ICP standards, and variable temperature MCD spectra for E17A/R47A-NiSOD, E17R/R47E-NiSOD, and R47A-NiSOD. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Figure 1.
The active sites of A) Cu/Zn-, B) Fe-, C) Mn-, and D) NiSOD.

Figure 2.

Overall hexamer (top left, looking down the 3-fold axis) and trimer (top right, perpendicular to the 3-fold axis) protein oligomeric state(s), and active site of NiSOD shown in the His-on, Ni(III)-bound state and H-bonding interactions from Glu17 and Arg47 (1T6U).

Figure 3.
DSC thermograms of H1A-, R47A-, E17A/R47A-, and E17R/R47E-NiSOD compared with WT-NiSOD.

Figure 4. Size exclusion chromatograms for WT- (black), H1A- (orange), R47A- (purple), E17A/R47A (pink), and E17R/R47E-NiSOD (green).

Figure 5.
EPR spectra of R47A- (purple) and E17R/R47E-NiSOD (green).

Figure 6. Ni K-edge XANES spectra for as-isolated WT- (black), H1A- (dark orange), and H1A_{IMD}-NiSOD (light orange). Insert: Pre-edge region showing features associated with $1s \rightarrow 3d$ and $1s \rightarrow 4p_z$ transitions.

Figure 7.

EXAFS spectra (colored lines) and best fits (black lines) from Table 4 for H1A- (dark orange) and H1A_{IMD}-NiSOD (light orange). Dashed line indicates fits generated without an imidazole ligand and solid line corresponds to fits including one imidazole ligand. The fitting procedure including coordinates from the **red** theoretical calculation was applied when no imidazole is present and the fitting procedure including coordinates from the **ox¹** theoretical calculation when an imidazole is present in the fit.

Figure 8. Ni K-edge XANES spectra for as-isolated WT- (black), R47A- (purple), E17A/R47A- (pink) and E17R/R47E-NiSOD (green). Insert: Pre-edge region showing features associated with $1s \rightarrow 3d$ and $1s \rightarrow 4p_z$ transitions.

Figure 9.

EXAFS spectra (colored lines) and fits (black lines) from Table 4 for R47A- (purple), E17A/R47A- (pink) and E17R/R47E-NiSOD (green). The fitting procedure including coordinates from the **ox¹** theoretical calculation was applied when an imidazole is present in the fit.

Figure 10.

The dependence of k_{cat} on pH for WT-, H1A-, R47A-, E17A/R47A-, and E17R/R47E-NiSOD. Conditions: 4uM H1A NiSOD, 30 mM formate, 10 mM phosphate, 5uM EDTA.

Figure 11. Spectral data taken during the reaction of WT- (black) and H1A-NiSOD (red, blue, and green) with pulse-radiolytically generated $\text{O}_2^{\bullet-}$. Conditions: 80uM H1A NiSOD, 30 mM formate, 10 mM phosphate, 5uM EDTA.

Table 1

Characterization of Native, WT, H1A, and H-bonding variants of NiSOD

NiSOD sample	MW (ESI-MS) (calc'd value)	Quaternary Structure (ESI-MS)	Ni/protein	Kinetics k _{cat} @ pH 7.5 ($\times 10^9 \text{ M}^{-1}\text{s}^{-1}$)	T _m (°C)
Native (<i>S. seoulensis</i>)	13,192.5 (13,189.1)	hexamer	0.74	1.18	88.0 ^b (>80)
WT	18,171.2 ^a (18,169.6)	hexamer	0.88	0.55	84.8 ^b 65.2 ^c
H1A-NiSOD	18,130.9 ^a (18,103.3)	hexamer	0.19	0.047	77.8 ^b
R47A-NiSOD	18,084.0 ^a (18,084.3)	hexamer	0.60	0.121	76.4 ^b
E17A/R47A-NiSOD	18,025.1 ^a (18,026.2)	hexamer	0.86	0.036	68.5 ^b
E17R/R47E-NiSOD	18,168.6 ^a (18,169.4)	hexamer	0.29	0.095	67.5 ^b

Table 2

Size-exclusion Chromatography

NiSOD sample	Elution volume (mL)	MW (kDa)	Corrected MW (kDa)	Quaternary Structure (cal'd, kDa)
WT	9.94	61.1	79.2	Hexamer (79.2)
H1A-NiSOD	10.08	58.9	76.5	Hexamer (78.8)
R47A-NiSOD	9.97	61.0	78.6	Hexamer (79.0)
	(12.76)	21.9	39.4	Trimer (39.5))
E17A/R47A-NiSOD	9.98	60.8	78.3	Hexamer (78.3)
	(12.47)	24.5	42.0	Trimer (39.2))
E17R/R47E-NiSOD	10.06	59.3	76.8	Hexamer (79.2)
	(12.59)	23.4	40.9	Trimer (39.6))

Table 3

XANES and EXAFS analysis of dithionite reduced and as-isolated WT-NiSOD using the proposed fitting model utilizing theoretical coordinates from ox¹ when an imidazole is included and red for when an imidazole is eliminated.

Sample	1s→3d peak area (×10 ² eV)	Geometry	N	R (Å)	σ^2 (×10 ³ Å ⁻²)	E ₀ (eV)	Reduced χ^2	Residual
Native NiSOD ^b Dithionite reduced	1.2 (7)	sqp	2N 2S	1.87(3) 2.15(1)	7(3) 1(1)	1(2)	9.53	0.0311
WT-NiSOD ^a as-isolated	3.5(9)	spy	2N IN 2S	1.73 (3) 2.09 (1) 2.18(6)	1 (1) 1 (12) 4 (4)	−14 (7)	40.43	0.0267
WT-NiSOD ^b as-isolated			2N IN 2S	1.74 (6) 1.9 (2) 2.11 (9)	1 (3) 0 (6) 4 (4)	−13 (2)	77.32	0.0468

^aIncludes one imidazole,

^bIncludes zero imidazole, pl- planar, py-square pyramidal

Table 4

XANES analysis and selected EXAFS fits for the H-bonding variants of NiSOD.

Sample	Edge Energy (eV)	1s→3d peak area ($\times 10^2$ eV)	Geometry	N	R(Å)	($\times 10^3$ Å ⁻²)	E ₀ (eV)	Residual
H1A-NiSOD ^b	8342.8	0.7 (2)	p1	2N 2S	1.91 (3) 2.18(1)	8 (2) 4 (1)	5 (2)	0.028
H1A _{IMD} -NiSOD ^a	8344.6	2.9 (6)	O _h	1N 3N 2S	1.89 (6) 2.07 (3) 2.28 (3)	6 (5) 3 (2) 8 (3)	5 (3)	0.016
H1A _{IMD} -NiSOD ^b				1N 3N 2S	1.9 (1) 2.08 (4) 2.29 (4)	8 (12) 11 (7) 9 (7)	7 (4)	0.033
H1A _{IMD} -NiSOD ^c				1N 3N 2S	1.81 (3) 2.03 (3) 2.25 (2)	3 (3) 4 (2) 6 (2)	2 (2)	0.031
R47A-NiSOD ^a	8344.4	2.5 (6)	py	1N 2N 2S	2.19 (7) 1.85 (4) 2.16 (1)	2 (4) 5 (2) 1 (4)	11 (7)	0.025
R47A-NiSOD ^b				1N 2N 2S	2.11 (8) 1.87 (4) 2.16 (8)	0 (10) 7 (6) 2 (2)	7 (11)	0.040
E17A/R47A-NiSOD ^a	8343.6	1.4 (6)	py	1N 2N 2S	1.97 (5) 1.81 (3) 2.19 (2)	0 (3) 4 (1) 4 (2)	1 (4)	0.037
E17A/R47A-NiSOD ^b				1N 2N 2S	1.95 (7) 1.79 (4) 2.18 (2)	1 (6) 3 (2) 3 (1)	2 (5)	0.038
E17R/R47E-NiSOD ^a	8343.6	3.2 (7)	py	1N 2N 2S	2.12 (3) 1.82 (4) 2.14 (4)	0 (2) 4 (2) 2 (8)	9 (7)	0.089
E17R/R47E-NiSOD ^b				1N 2N 2S	2.08 (3) 1.83 (5) 2.15 (3)	0 (9) 5 (3) 4 (12)	4 (9)	0.072