Published in final edited form as:

J Biol Inorg Chem. 2010 June ; 15(5): 777–793. doi:10.1007/s00775-010-0641-2.

Spectroscopic and computational investigation of three Cys-to-Ser mutants of nickel superoxide dismutase: insight into the roles played by the Cys2 and Cys6 active-site residues

Olivia E. Johnson,

Department of Chemistry, University of Wisconsin-Madison, 1101 University Avenue, Madison, WI 53706, USA

Kelly C. Ryan,

Department of Chemistry, University of Massachusetts at Amherst, 104 Lederle Graduate Research Tower A, 710 North Pleasant Street, Amherst, MA 01003, USA

Michael J. Maroney, and

Department of Chemistry, University of Massachusetts at Amherst, 104 Lederle Graduate Research Tower A, 710 North Pleasant Street, Amherst, MA 01003, USA

Thomas C. Brunold

Department of Chemistry, University of Wisconsin-Madison, 1101 University Avenue, Madison, WI 53706, USA

Thomas C. Brunold: brunold@chem.wisc.edu

Abstract

Nickel-dependent superoxide dismutase (Ni-SOD) is a member of a class of metalloenzymes that protect aerobic organisms from the damaging superoxide radical ($\text{O}_2^{\cdot-}$). A distinctive and fascinating feature of NiSOD is the presence of active-site nickel–thiolate interactions involving the Cys2 and Cys6 residues. Mutation of one or both Cys residues to Ser prevents catalysis of $\text{O}_2^{\cdot-}$, demonstrating that both residues are necessary to support proper enzymatic activity (Ryan et al., *J Biol Inorg Chem*, 2010). In this study, we have employed a combined spectroscopic and computational approach to characterize three Cys-to-Ser (Cys $\rightarrow$ Ser) mutants (C2S, C6S, and C2S/C6S NiSOD). Similar electronic absorption and magnetic circular dichroism spectra are observed for these mutants, indicating that they possess nearly identical active-site geometric and electronic structures. These spectroscopic data also reveal that the Ni^{2+} ion in each mutant adopts a high-spin ($S = 1$) configuration, characteristic of a five- or six-coordinate ligand environment, as opposed to the low-spin ($S = 0$) configuration observed for the four-coordinate Ni^{2+} center in the native enzyme. An analysis of the electronic absorption and magnetic circular dichroism data within the framework of density functional theory computations performed on a series of five- and six-coordinate C2S/C6S NiSOD models reveals that the active site of each Cys $\rightarrow$ Ser mutant possesses an essentially six-coordinate Ni^{2+} center with a rather weak axial bonding interaction. Factors contributing to the lack of catalytic activity displayed by the Cys $\rightarrow$ Ser NiSOD mutants are explored.

Correspondence to: Thomas C. Brunold, brunold@chem.wisc.edu.

Electronic supplementary material The online version of this article (doi:10.1007/s00775-010-0641-2) contains supplementary material, which is available to authorized users.

Keywords

Nickel-dependent superoxide dismutase; Redox-active nickel enzymes; Magnetic circular dichroism; Density functional theory

Introduction

The superoxide radical anion ($\text{O}_2^{\cdot-}$), which is formed during aerobic metabolism, causes oxidative damage of several biological molecules [1–6]. As a result, $\text{O}_2^{\cdot-}$ has been implicated in a number of medical conditions and disorders, including Alzheimer's and Parkinson's diseases, certain cancers, familial amyotrophic lateral sclerosis, and aging in general [7–15]. In an effort to reduce the concentration of $\text{O}_2^{\cdot-}$, nature has evolved a family of metalloenzymes known as superoxide dismutases (SODs) that disproportionate $\text{O}_2^{\cdot-}$ to dioxygen (O_2) and hydrogen peroxide (H_2O_2) via a two-step ping-pong catalytic mechanism in which the metal center (M) cycles between reduced and oxidized states (Eqs. 1,2) [3,16–18].

To date, three distinct classes of SODs, distinguished by their different redox-active metal centers, have been thoroughly investigated; namely, Fe-, Mn-, and Cu, Zn-SOD [17,19,20]. In comparison, relatively little is known about Ni-dependent superoxide dismutase (NiSOD), which is the most recently discovered member of the SOD family. Nevertheless, research carried out in the last decade revealed that NiSOD is strikingly different from the other SOD members in terms of its spectroscopic properties, active-site ligand environment, and amino acid sequence. This enzyme, found in *Streptomyces* species [21,22] and several cyanobacteria [23], is typically a homohexamer of approximately 13 kDa monomers, each containing one Ni atom [24,25]. The Ni center, located at the N terminus of the protein, is held in place by the “Ni-hook” motif, which consists of six conserved amino acid residues. Within this Ni-hook, the Ni^{3+} ion of the oxidized enzyme (NiSOD_{ox}) is found in a five-coordinate, square-pyramidal ligand environment with the imidazole donor from a histidine (His1) in the axial position and two *cis*-thiolates from cysteine residues (Cys2 and Cys6), a deprotonated amide from the Cys2 backbone, and the N-terminal $-\text{NH}_2$ group of His1 in the equatorial positions (Fig. 1) [24,25]. In the reduced Ni^{2+} -bound state ($\text{NiSOD}_{\text{red}}$), the distance between Ni and His1 is significantly larger, resulting in a square-planar ligand environment of the Ni^{2+} ion [24,25].

Compared with other SOD enzymes, NiSOD is unique. With the exception of Cu, Zn-dependent SOD, NiSOD is the only member of the SOD family in which the coordination number changes as a function of metal ion oxidation state [26]. Moreover, NiSOD's active-site environment is strikingly different from those observed for the other SODs as well as related metalloenzymes. Although coordination by deprotonated amides is observed for several metalloproteins [27,28], ligation by the N-terminal amine has been established in only very few cases [29,30], such as the CO sensor protein CoxA [31]. Ligation by the equatorial Cys thiolates in NiSOD is remarkable, as these residues are susceptible to oxidation by derivatives of $\text{O}_2^{\cdot-}$. The susceptibility of thiolates to strong oxidants has been

demonstrated by the immediate oxidation of these highly nucleophilic ligands by oxygenation [32–39], alkylation [33,37,38,40,41], and protonation [42] in various synthetic $\text{Ni}^{2+}\text{-N}_x\text{S}_y$ species. Nevertheless, previous studies have shown that the redox activity of NiSOD is metal centered, not ligand-based [43–45].

Although thiolate ligation in NiSOD is distinct among the SOD family, it has also been observed in four other crystallographically characterized redox-active Ni-containing metalloenzymes [46], including [NiFe] hydrogenases [47], CO dehydrogenases [48], acetyl CoA synthase [27], and methyl coenzyme M reductase [49]. In contrast, a variety of Ni-binding proteins and Ni-containing metalloenzymes featuring a N/O ligand environment are redox-inactive [46,50–53]. On this basis, the unusual coordination by Cys2 and Cys6 was proposed to properly tune the reduction potential of NiSOD [54]. In fact, the metal ion reduction potential of wild-type NiSOD, $E^\circ[\text{Ni}^{3+}/\text{Ni}^{2+}] = 290 \text{ mV}$ (vs. the normal hydrogen electrode) [55], lies approximately midway between the potentials for the oxidation and reduction of $\text{O}_2^{\cdot-}$ ($E^\circ = 160$ and 890 mV vs. the normal hydrogen electrode, respectively) [56]. It has been demonstrated that Ni complexes with N/O ligation have considerably higher $\text{Ni}^{3+/2+}$ potentials, but substitution with anionic S-donor ligands lowers E° significantly, suggesting that thiolate ligands in redox-active Ni-containing metalloenzymes are necessary for catalytic activity [57–59].

In a manner similar to that of other SODs, the protein environment surrounding the Ni ion influences the enzyme activity. Computational work by Fiedler et al. [43] led to the suggestion that the Ni–thiolate bonding interactions are crucial with respect to the activity of NiSOD. Filled–filled π interactions between occupied Ni $3d$ (xz/yz) and S/N π -based orbitals from the thiolates and the deprotonated amide result in destabilized redox-active molecular orbitals (MOs), thereby facilitating electron transfer to $\text{O}_2^{\cdot-}$ and promoting Ni^{2+} -based oxidation over S-based oxidation. Because the Ni–thiolate bonds are seemingly incompatible with the NiSOD catalytic reaction, an outer-sphere mechanism was proposed as opposed to an inner-sphere mechanism for the disproportionation of $\text{O}_2^{\cdot-}$. However, the molecular mechanism for the reduction and oxidation of $\text{O}_2^{\cdot-}$ employed by NiSOD is still under debate. In an effort to settle this issue, azide (N_3^-), a popular $\text{O}_2^{\cdot-}$ analogue, was used to assess whether substrate can bind directly to the metal center of NiSOD. This anion is particularly useful, as it is similar to $\text{O}_2^{\cdot-}$ in size, charge, and nature of the frontier orbitals [60,61]. An electron paramagnetic resonance (EPR) study carried out by Barondeau et al. [24] demonstrated that as-isolated NiSOD produced fine structure in the g_y region with ^{14}N -labeled azide. This region was unaffected with ^{15}N -labeled azide, precluding a direct interaction between the anion and the Ni center, thus arguing against inner-sphere binding. Alternatively, a recent study by Tietze et al. [62] revealed that cyanide can access the Ni center of a NiSOD_{red} peptide that exhibits an absorption spectrum similar to that of the native enzyme, which prompted the authors to suggest inner-sphere binding of substrate during catalysis.

To evaluate the importance of the active-site Cys thiolates with respect to the NiSOD properties, Ryan et al. [63] studied the kinetic and X-ray spectroscopic properties of three NiSOD mutants in which one or both Cys residues were mutated to Ser (C2S, C6S, C2S/C6S NiSOD). Their results revealed that a single Cys-to-Ser (Cys $\rightarrow$ Ser) mutation is sufficient to cause a complete loss of SOD activity, highlighting the importance of these Cys residues. However, the lack of X-ray crystal structures for any of the Cys $\rightarrow$ Ser NiSOD mutants precluded an interpretation of these results in terms of altered active-site geometric and electronic properties of the variants. To address this issue, we have used electronic absorption, circular dichroism (CD), and magnetic CD (MCD) spectroscopies in conjunction with density functional theory (DFT) and time-dependent DFT (TD-DFT) calculations to explore the active-site properties of the three Cys $\rightarrow$ Ser NiSOD mutants. Our

computational method was validated by carrying out parallel calculations on the H134A urease mutant [64], which exhibits MCD spectral features strikingly similar to those of the Cys → Ser NiSOD mutants. The spectroscopic and computational results obtained in this study provide significant insight into the factors contributing to the lack of SOD activity displayed by these three mutants.

Materials and methods

Sample preparation

The expression, purification, and metallation of the C2S, C6S, and C2S/C6S NiSOD variants were carried out as described in [63]. For room temperature electronic absorption and CD studies, protein samples were prepared in 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.5). Samples for the CD and MCD studies were prepared in approximately 55% (v/v) mixtures of glycerol and either 50 mM Tris or 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer. The final Ni concentrations for C2S, C6S, and C2S/C6S NiSOD in 50 mM Tris buffer were 4.14, 4.50, and 4.01 mM, respectively. The azide-treated C2S/C6S NiSOD sample was prepared by the addition of 56 μ L of an aqueous NaN₃ solution (5 M, 50 mM Tris) to 140 μ L of C2S/C6S NiSOD (10 mM), corresponding to an N₃[−] to Ni ratio of approximately 200:1. The azide-treated C2S and C6S NiSOD samples were prepared by the addition of 22.4 μ L of an aqueous NaN₃ solution (5 M, 50 mM Tris) to 140 μ L of C2S and C6S NiSOD (10 mM), corresponding to an N₃[−] to Ni ratio of approximately 80:1. The final Ni concentrations for the C2S, C6S, and C2S/C6S NiSOD azide-treated samples were 3.58, 4.88, and 2.49 mM, respectively. For low-pH studies of the Cys → Ser NiSOD mutants, the 50 mM Tris buffer (pH 8.5) was exchanged for 50 mM MES buffer (pH 5.5) by centrifugation. The final Ni concentrations for the C2S, C6S, and C2S/C6S NiSOD samples at low pH were 2.7, 2.2, and 3.2 mM, respectively.

Spectroscopy

Room temperature electronic absorption spectra were collected with a Varian Cary 5E spectrophotometer. Low and room temperature CD data were obtained using a JASCO J-715 spectropolarimeter. Low temperature MCD spectra were collected using the JASCO J-715 instrument in conjunction with an Oxford Instruments SM-4000 8 T superconducting magnetocryostat at 4, 8, 15, 30, and 50 K. CD and glass-strain contributions to the MCD spectra were removed by subtracting the −7 T data from the +7 T data.

Computations

The Amsterdam Density Functional 2006.01 software package was used to perform spin-unrestricted DFT geometry optimizations [65–69] of the active-site models described later. These computations were carried out using Amsterdam Density Functional triple- ζ Slater-type orbitals with a single set of polarization functions (basis set IV), an integration constant of 4.0, and the Vosko et al. [70] local density approximation, along with the nonlocal gradient corrections of Becke [71] and Perdew [72]. The core orbitals were frozen through 1s (C, N, and O) and 2p for Ni.

H134A urease active-site model—Initial coordinates for the active-site model of the crystallographically characterized H134A urease mutant were obtained from the Protein Data Bank (file 1FWI) [64]. The active-site model consisted of the central Ni ion, three-coordinated water molecules, a carbamylated lysine, and two His ligands. The amino acid residues were truncated at the α -carbon, and hydrogen atoms were added to convert these α -carbons into terminal methyl groups. The model was subjected to a constrained-geometry optimization in which the positions of all of the α -carbons were kept fixed, whereas the

remaining atoms were allowed to move freely. The Cartesian coordinates for the H134A urease model are included in the Supporting Information (Table S10).

C2S/C6S NiSOD active-site models—Initial coordinates for the five-coordinate C2S/C6S^{5-c} and six-coordinate C2S/C6S^{6-c} active-site models were derived from the crystal structure of wild-type NiSOD_{ox} deposited in the Protein Data Bank (file 1TU6) [24]. In all protein active-site models considered, the amino acid residues were truncated at the α -carbon, with the exception of His1, which was modeled as an imidazole, and hydrogen atoms were added to convert the terminal carbon atoms into methyl groups. The coordinates of these terminal methyl groups were held fixed throughout the geometry optimizations, whereas all other atoms were allowed to move freely. To generate the perturbed C2S/C6S^{6-c} models used to reconcile our MCD and DFT computational results with the X-ray absorption spectroscopy (XAS) data presented in [63] (see “Discussion”), the Ni–OH₂ bond distance in the DFT geometry-optimized C2S/C6S^{6-c} active-site model was systematically lengthened in fixed increments of 0.15 Å from the unperturbed distance of 2.57 Å, whereas the coordinates of all other atoms were kept fixed. The Cartesian coordinates for the DFT geometry optimized C2S/C6S^{5-c} and C2S/C6S^{6-c} NiSOD active-site and perturbed C2S/C6S^{6-c} models mentioned in the text are provided in the Supporting Information (Tables S11–S15).

Intermediate neglect of differential overlap/spectroscopic parameterization with configuration interaction calculations

Semiempirical intermediate neglect of differential overlap/spectroscopic parameterization with configuration interaction (INDO/S-CI) calculations were carried out using the ORCA 2.4 software package developed by Neese [73]. This program uses the model of Ridley and Zerner [74] and Zerner et al. [75], the valence shell ionization potentials and Slater–Condon parameters listed by Bacon and Zerner [76], and the standard interaction factors $f_{pp\sigma\sigma} = 1.266$ and $f_{pp\sigma\sigma} = 0.585$. Restricted open-shell Hartree–Fock self-consistent-field calculations were tightly converged on the $S = 1$ ground state, which then served as the reference state for configuration interaction calculations. Calculations of electronic transition energies and intensities included single excitations from all doubly occupied MOs (DOMOs) within 10 eV of the highest occupied MO (HOMO) to the two singly occupied MOs (SOMOs) and the unoccupied MOs within 19 eV of the lowest occupied MO. Double excitations included the 32 highest energy DOMOs, the 2 SOMOs, and 6 virtual MOs. Larger active spaces did not significantly alter the calculated parameters.

TD-DFT calculations

The TD-DFT calculations for all protein active-site models were performed in ORCA using Becke’s three-parameter hybrid functional for exchange combined with the Lee–Yang–Par correlation functional (B3LYP) [71,77,78]. The SV(P) (Ahlrichs’s polarized split valence) basis [79] with the SV/C auxiliary basis [80] was used for all atoms except for Ni, which was described with the TZV(P) (Ahlrichs’s polarized triple- ζ valence) basis [81]. Isosurface plots of the MOs were generated with the gOpenMol program using an isodensity of value of 0.045 a.u. [82].

Results

Spectroscopic results

The room temperature electronic absorption spectra of C2S, C6S, and C2S/C6S NiSOD (Fig. 2) are strikingly different from the electronic absorption spectrum of wild-type NiSOD reported previously [21,22,43]. The spectrum of each mutant is dominated by a broad feature in the near-UV region at approximately 25,000 cm^{−1} ($\epsilon = 90\text{--}190 \text{ M}^{-1} \text{ cm}^{-1}$), with a

high-energy shoulder around 28,000 cm⁻¹ region ($\epsilon = 60\text{--}200\text{ M}^{-1}\text{ cm}^{-1}$). Toward lower energy, a weaker broad feature is observed near 16,000 cm⁻¹ ($\epsilon = 8\text{--}20\text{ M}^{-1}\text{ cm}^{-1}$). The major electronic absorption features of the Cys $\rightarrow$ Ser NiSOD mutants agree well with those reported for high-spin ($S = 1$) Ni²⁺ chromophores [83–87]. The MCD spectra of the mutants (Fig. 3) display three intense, negatively signed features at approximately 9,500, 15,500, and 28,000 cm⁻¹, along with very weak negatively signed bands at about 13,000, 17,000, and 24,000 cm⁻¹. The entire spectrum exhibits temperature-dependent C-term behavior, verifying that each observed feature is associated with a high-spin Ni²⁺ center [88]. This finding explains the lack of an EPR signal displayed for the Cys $\rightarrow$ Ser NiSOD mutants [63]. High-spin Ni²⁺ species typically exhibit a rather large zero-field splitting, preventing the use of conventional X-band EPR measurements, thus rendering the Ni²⁺ ion “EPR-silent” [89]. Interestingly, the strong resemblance of the electronic absorption, CD (Fig. S1), and MCD spectra obtained for all three mutants indicates nearly identical active-site environments. Although this result was rather unexpected, it is consistent with the similarity of the Ni²⁺ 1s $\rightarrow$ 3d transition energies and intensities of these mutants in the X-ray absorption spectra obtained by Ryan et al. [63].

Typically, paramagnetic Ni²⁺ complexes possess either a five-coordinate or a six-coordinate coordination environment that can be distinguished by electronic absorption spectroscopy on the basis of the energies of the three spin-allowed ligand-field (LF) transitions from the ³A_{2g} ground state to the ³T_{2g}, ³T_{1g}(F), and ³T_{1g}(P) excited states (using symmetry labels from the O_h parent point group) [87]. It is well known that six-coordinate Ni²⁺ systems display three electronic absorption bands in the ranges 7,000–13,000, 11,000–20,000, and 19,000–28,000 cm⁻¹, corresponding to ³A_{2g} $\rightarrow$ ³T_{2g}, ³A_{2g} $\rightarrow$ ³T_{1g}(F), and ³A_{2g} $\rightarrow$ ³T_{1g}(P) LF transitions, respectively [84–87,90]. Alternatively, for five-coordinate Ni²⁺ species, the ³A_{2g} $\rightarrow$ ³T_{2g} LF transition shifts below 7,000 cm⁻¹, whereas the ³A_{2g} $\rightarrow$ ³T_{1g}(F) and ³A_{2g} $\rightarrow$ ³T_{1g}(P) LF transitions remain between 7,000 and 25,000 cm⁻¹ [83,87,91–94]. Consequently, the presence of only two broad, near-UV/vis features in the electronic absorption spectra of the Cys $\rightarrow$ Ser mutants as well as the relatively high intensities of the major electronic absorption band ($\epsilon > 30\text{ M}^{-1}\text{ cm}^{-1}$) appear to favor a five-coordinate active site [87]. This hypothesis is supported by the X-ray absorption near edge structure data obtained by Ryan et al. [63], given that the Ni 1s $\rightarrow$ 3d peak areas are in the range 0.059–0.067 eV for the Cys $\rightarrow$ Ser mutants. However, although the lack of a discernible near-IR electronic absorption feature may suggest that the mutants possess a five-coordinate active site, it is possible that the intensity of the ³A_{2g} $\rightarrow$ ³T_{2g} transition is too weak to produce a noticeable electronic absorption feature.

Because MCD spectroscopy offers a considerably more sensitive probe of paramagnetic metal centers than electronic absorption spectroscopy, the former provides a better method for determining the number and energies of the spin-allowed LF transitions for high-spin Ni²⁺ species. Indeed, the MCD spectra reported for a variety of well-characterized high-spin Ni²⁺ complexes [64,95–103] exhibit three intense temperature-dependent bands due to the ³A_{2g} $\rightarrow$ ³T_{2g}, ³T_{1g}(F), and ³T_{1g}(P) LF transitions, whereas the electronic absorption spectra often lack a distinct feature associated with the ³A_{2g} $\rightarrow$ ³T_{2g} transition. Similarly, the MCD spectra of the Cys $\rightarrow$ Ser NiSOD mutants display three intense bands that fall within the ranges for the ³A_{2g} $\rightarrow$ ³T_{2g}, ³A_{2g} $\rightarrow$ ³T_{1g}(F), and ³A_{2g} $\rightarrow$ ³T_{1g}(P) transitions, thus providing evidence for a six-coordinate Ni²⁺ center as opposed to a five-coordinate metal ion environment. The presence of additional weak bands in the MCD spectra is indicative of a splitting of the three LF excited states observed for rigorously octahedral Ni²⁺ species, consistent with the expectation that the mutants possess a distorted six-coordinate active site [103].

Gaussian deconvolutions—To determine the number and energies of the transitions contributing to the electronic absorption, CD, and MCD spectra of the Cys → Ser NiSOD mutants, we performed a simultaneous Gaussian deconvolution of these spectra. Only the spectral analysis for the C2S/C6S NiSOD mutant is presented here (Fig. 4, Table 1), since virtually identical results were obtained for the C2S and C6S NiSOD variants (Figs. S2, S3, Tables S1, S2). A minimum of 11 Gaussian bands were required to achieve satisfactory agreement between the simulated and experimental spectra. On the basis of their large ratios of MCD intensity to electronic absorption intensity ($|\Delta\epsilon|/\epsilon$ ratios), bands 2–4, 8, and 9 can be attributed to transitions primarily involving Ni 3d-based MOs (note that the $|\Delta\epsilon|/\epsilon$ ratios for bands 8 and 9 are considerably lower than those for bands 2–4, consistent with the results reported for other high-spin Ni^{2+} complexes) [96]. Although the $|\Delta\epsilon|/\epsilon$ ratio for band 1 could not be determined, a LF transition is likely responsible for this band given its low energy and high MCD intensity. Alternatively, bands 5 and 6, which correspond to the intense, oppositely signed CD features that produce the dominant contributions to the electronic absorption spectrum, possess significantly lower $|\Delta\epsilon|/\epsilon$ ratios and therefore are attributed to transitions possessing primarily charge transfer character with negligible contributions from the Ni 3d orbitals. The small $|\Delta\epsilon|/\epsilon$ ratios found for bands 10 and 11 also suggest that the corresponding transitions have mainly charge transfer character. Lastly, the narrow width, low MCD intensity, and absence in the electronic absorption spectrum indicate that band 7 is due to a spin-forbidden LF transition [103].

Low-pH study—In the wild-type NiSOD crystal structure, two ordered water molecules are observed near the vacant sixth position, approximately 4.0 Å and approximately 3.6 Å away from the metal center in the Ni^{3+} and Ni^{2+} states [24,25]. Consequently, it is conceivable that in the Cys → Ser Ni-SOD mutants, an expansion of the active-site coordination environment occurs by binding of solvent to the high-spin Ni^{2+} ion. Varying the solvent pH of a metalloenzyme while monitoring relevant spectral features is a simple yet powerful technique used to probe the coordination of water molecules to a metal center. Therefore, this approach was employed in the present study to explore if solvent occupies the vacant position in the mutants' active site. Because the stability of wild-type NiSOD is maintained over the pH range of approximately 4.0–8.0, the Cys → Ser NiSOD mutants were also studied at a pH of 5.5 (this value was chosen to ensure coordination of Ni to the corresponding enzyme active sites) [21]. On lowering the pH from 8.5 to 5.5, we observed minor to no changes in the electronic absorption and CD spectra of each of the Cys → Ser Ni-SOD variants investigated (Figs. S4–S7). However, as demonstrated already, MCD spectroscopy provides a much more sensitive probe of the ligand environment of high spin Ni^{2+} centers. Indeed, a comparison of the MCD spectra of the Cys → Ser NiSOD mutants obtained at pH 8.5 and 5.5 (Fig. 5) reveals small, but discernable shifts not detected in the electronic absorption and CD spectra. The effect of lowering the pH on the Ni^{2+} LF transitions was assessed by conducting a simultaneous Gaussian deconvolution of the mutants' electronic absorption, CD, and MCD spectra at pH 5.5 (Figs. S8–S10, Tables S3–S5). This analysis revealed a red shift of the LF transitions associated with bands 1–4, 8, and 9 of the C2S and C6S NiSOD mutants and bands 1, 2, and 4 for C2S/C6S NiSOD, with the most notable shifts involving bands 1, 2, and 4 in each case (Table S6). Although the similarity of the MCD data of the Cys → Ser NiSOD mutants at pH 8.5 suggests nearly identical Ni^{2+} coordination environments (vide supra), the subtle differences in the MCD spectral shifts between the single and double mutants in response to lowering the pH provides further evidence that in each species the Ni^{2+} ion does indeed occupy the active site observed in wild-type NiSOD. Although the number of bands affected by lowering the pH differs among the single and double mutants, the red shifts likely reflect a significant lengthening of a Ni^{2+} –ligand bond and thus suggest the presence of coordinated solvent at the active sites of all three mutants. Indeed, similar band shifts were observed for synthetic

six-coordinate ($S = 1$) Ni^{2+} complexes upon weakening of an axial ligand– Ni^{2+} bonding interaction [84,104]. Note that in the case of the Cys $\rightarrow$ Ser NiSOD mutants, the Ni–OH₂ bond could also be perturbed via a second-sphere effect, such as protonation of a residue involved in a hydrogen-bond interaction with the coordinated solvent.

Further support for the coordination of a solvent molecule to the Ni^{2+} ions of the Cys $\rightarrow$ Ser NiSOD mutants was obtained by comparing the corresponding CD spectra obtained at room temperature and at 4 K (Fig. 6). In each case, lowering the temperature causes the oppositely signed features at 21,500 and 24,200 cm^{-1} to blue shift by approximately 200 and 50 cm^{-1} , respectively. These band shifts are consistent with a slight structural rearrangement of the mutants' active sites, possibly involving a tighter binding of a solvent molecule to the Ni^{2+} center. Consistent with this hypothesis, modulating the temperature was shown to have a strong effect on the coordination of solvent in octahedral ($S = 1$) Ni^{2+} complexes with $\text{X}_2\text{Y}_2\text{Z}_2$ coordination spheres (where Z is water or another solvent molecule) [101,102,105–107]. These species generally exhibit significant thermochromic behavior owing to a change in coordination number from 6 to 4 with increasing temperature, which gives rise to dramatic changes in the corresponding electronic absorption and MCD spectra. In the case of the Cys $\rightarrow$ Ser NiSOD mutants, the thermochromic behavior is much less pronounced as the Ni^{2+} ion likely remains at least five-coordinate at room temperature and thus maintains an $S = 1$ ground state.

Substrate analogue study—All SODs catalyze the disproportionation of $\text{O}_2^{\cdot-}$ at rates that are at or near the diffusion limit [18,19], which prevents substrate–SOD intermediates from being trapped and studied. Therefore, small anion analogues of $\text{O}_2^{\cdot-}$ are commonly employed to explore the active site/ $\text{O}_2^{\cdot-}$ interactions in SODs. For wild-type NiSOD, it was initially proposed that $\text{O}_2^{\cdot-}$ coordinates directly to the Ni center [24]. However, on the basis of spectroscopic studies using the substrate analogue N_3^- , a close electronic mimic of $\text{O}_2^{\cdot-}$, it was concluded that the substrate does not actually have access to the NiSOD active site. Specifically, only minor shifts were observed in the MCD spectrum upon N_3^- -treatment of NiSOD_{ox}, ruling out the formation of an inner-sphere complex in the Ni^{3+} state [43]. Likewise, the Shearer laboratory [108] used the absence of noticeable shifts in the electronic absorption spectrum as evidence for the inability of N_3^- to access the Ni^{2+} center in a wild-type-like NiSOD_{red} peptide (consisting of only the Ni-hook motif residues). Although these earlier studies demonstrated that azide is incapable of binding to the metal center in wild-type NiSOD, they did not provide any definitive clues as to whether formation of an inner-sphere complex is hindered by (1) the ligand environment imposed on the Ni ion and/or (2) second-sphere residues. Since the Ni^{2+} ions of the Cys $\rightarrow$ Ser NiSOD mutants are likely coordinated by an easily displaceable axial water molecule (vide supra), they should also be accessible to small anions. Therefore, studies of the C2S, C6S, and C2S/C6S NiSOD mutants provide a unique opportunity for developing a better understanding of why N_3^- coordination to the Ni^{2+} ion of wild-type NiSOD is inhibited.

Addition of 80–100 equiv of N_3^- to the Cys $\rightarrow$ Ser NiSOD mutants resulted in virtually no changes in the electronic absorption and CD spectra (Figs. S11–S14). However, small but noticeable shifts are observed in the mutants' MCD spectra (Fig. 7). The relative band shifts are similar in each case, suggesting that the addition of azide has essentially the same effect on all three Cys $\rightarrow$ Ser NiSOD mutants. Importantly, the small magnitude of these shifts argues against a direct interaction of this anion with the Ni^{2+} ion. Given the strong possibility that an easily displaceable water molecule resides within the active site of the three Cys $\rightarrow$ Ser NiSOD mutants, this finding suggests that coordination of small anions, such as N_3^- , to the Ni center may be prevented by second-sphere residues. A recent study by Herbst et al. [55] of the Y9F NiSOD mutant in the presence of Cl^- and Br^- revealed an anion binding pocket consisting of the Tyr9 hydroxyl group and the amide hydrogens of the

Cys6 and Asp3 backbones. On the basis of our spectroscopic data, it is quite likely that N_3^- coordinates to a similar anion binding pocket in the Cys $\rightarrow$ Ser NiSOD mutants.

Computational results

Validation of computational approach—As mentioned earlier, our electronic absorption and MCD data appear to favor five- and six-coordinate active-site environments for the Cys $\rightarrow$ Ser NiSOD mutants, respectively. Therefore, DFT and TD-DFT calculations were performed to optimize the geometries and predict the transition energies and electronic absorption intensities, respectively, for five- and six-coordinate active-site models. To validate the use of this computational approach, we performed analogous DFT and TD-DFT calculations on the structurally characterized active site of H134A urease, which contains a single, high-spin ($S = 1$) Ni^{2+} ion in a distorted O_h ligand environment. In analogy to the MCD data obtained for the Cys $\rightarrow$ Ser NiSOD mutants, three negatively signed, temperature-dependent features, corresponding to the ${}^3\text{A}_{2g} \rightarrow {}^3\text{T}_{2g}$, ${}^3\text{A}_{2g} \rightarrow {}^3\text{T}_{1g}(\text{F})$, and ${}^3\text{A}_{2g} \rightarrow {}^3\text{T}_{1g}(\text{P})$ LF transitions of octahedral ($S = 1$) Ni^{2+} centers, dominate the MCD spectrum of H134A urease [64]. This close similarity between the MCD spectral features of the H134A urease and Cys $\rightarrow$ Ser NiSOD mutants suggests a similar six-coordinate Ni^{2+} ligand environment in all these species.

A constrained DFT geometry optimization carried out on the active site of H134A urease yielded a distorted O_h Ni^{2+} center (Fig. 8), consistent with structural and spectroscopic data for this mutant. However, the optimized Ni–N/O distances deviate significantly from those determined by X-ray crystallography (Table S7). This is not surprising, though, as the average Ni–N/O distance observed in the X-ray crystal structure (2.31 Å) differs markedly from that obtained by extended X-ray absorption fine structure (EXAFS) studies (2.09 Å) of H134A urease, which likely reflects the low resolution (2.0 Å) of the crystal structure [64]. Importantly, the average Ni–N/O distance in the DFT-optimized structure (2.14 Å) agrees very well with the distance determined by the EXAFS analysis. The optimized Ni–N distances for the H134A urease active-site model are also in good agreement with those reported for structurally similar, synthetic high-spin Ni^{2+} complexes, whereas the Ni–O bond lengths are overestimated by approximately 0.14–0.2 Å [84,86,109–114].

The TD-DFT-computed LF transition energies for the optimized H134A urease active-site model are presented in Table S8, along with those obtained experimentally from an MCD spectroscopic study of H134A urease [64]. This comparison reveals that the TD-DFT method underestimates the highest LF transition energy by approximately $8,000\text{ cm}^{-1}$, possibly because the single-determinantal wave function employed by DFT does not accurately describe the LF excited states of high-spin Ni^{2+} complexes [115]. The LF transition energies were therefore also calculated using the semiempirical INDO/S-CI method, which permits a multideterminantal representation of the excited states [75]. As shown in Table S8, the INDO/S-CI approach leads to a significant increase of the highest LF transition energy and thus yields better agreement with the experimental results. Regardless of the computational method used, however, the calculated LF transition energies for H134A urease are in relatively good agreement with those determined experimentally.

Cys $\rightarrow$ Ser NiSOD active-site models—Collectively, the close resemblance of the spectral data obtained for the Cys $\rightarrow$ Ser NiSOD single and double mutants not only suggests nearly identical active-site structures for all three species, but also implies that the remaining Cys residue in C2S and C6S NiSOD does not coordinate to the Ni^{2+} ion. Because the positioning of this remaining Cys residue in the single mutants is unknown, computational studies were performed on hypothetical five- and six-coordinate active-site models for C2S/C6S NiSOD. In the five-coordinate C2S/C6S NiSOD model (C2S/C6S^{5-c}),

the S atoms of the Cys2 and Cys6 residues were replaced by hydroxyl groups to introduce the Cys → Ser mutations (Scheme 1). Addition of an axial water molecule *trans* to the His1 imidazole ligand of C2S/C6S^{5-c} produced the six-coordinate C2S/C6S NiSOD model (C2S/C6S^{6-c}) (Scheme 1). Note that given the relatively high pK_a of free Ser of 13.6 [116], it seems likely that both Ser2 and Ser6 are in their neutral (protonated) forms in the C2S/C6S NiSOD mutant. Strong support for this assumption is provided by the good agreement between the experimental and computed transition energies for active-site models containing neutral Ser residues (*vide infra*).

The structural parameters for each geometry-optimized C2S/C6S NiSOD model are fairly reasonable (Table S9). Consistent with the MCD data, DFT predicts a distorted O_h Ni^{2+} center for the six-coordinate C2S/C6S^{6-c} model. The optimized Ni–N bond distances for this model agree well with those observed in related high-spin Ni^{2+} complexes [84,110,112–114]. However, the optimized Ni–O bond distances deviate significantly from those reported for structurally similar Ni^{2+} complexes, in particular the Ni–O_{H2O} bond length [84,110,112–114,117]. Although the Ni–O_{H2O} bond distance is longer than expected (approximately 2.6 Å), it is important to note that a similarly long Ni–O_{H2O} bond was obtained upon geometry optimization of the H134A urease active-site model (*vide supra*). For the five-coordinate C2S/C6S^{5-c} model, the DFT geometry optimization yielded a distorted square-pyramidal Ni^{2+} center. As expected, removal of the solvent molecule from C2S/C6S^{6-c} to generate C2S/C6S^{5-c} causes a slight increase in the N(His1)–Ni–N and N(His1)–Ni–O bond angles. The optimized bond angles and lengths for C2S/C6S^{5-c} are consistent with those of similar square-pyramidal $S = 1$ Ni^{2+} complexes, although the Ni–O bond distances in this model are, again, longer than those determined experimentally for synthetic complexes [84,118–120].

The electronic structures of the two C2S/C6S NiSOD active-site models were analyzed using the spin-unrestricted DFT method in conjunction with B3LYP. Tables 2 and 3 summarize the computed MO energies and compositions for C2S/C6S^{5-c} and C2S/C6S^{6-c}, respectively, and the corresponding MO diagrams are presented in Fig. 9. For both models, spin-polarization stabilizes the Ni *d*-based spin-up MOs relative to their spin-down counterparts, which leads to extensive mixing of the former with the ligand-based MOs. Therefore, for ease of analysis, only the spin-down MOs are considered here.

In agreement with the experimental data for the Cys → Ser NiSOD mutants, calculations for both the C2S/C6S^{5-c} and the C2S/C6S^{6-c} models predict that the two lowest energy unoccupied spin-down MOs derive from the Ni $d(z^2)$ (MOs 78 and 83) and Ni $d(x^2 - y^2)$ (MOs 79 and 84) orbitals. The HOMO and HOMO-1 are largely amide-based and carry a considerable amount of orbital character from the amide N and adjacent carbonyl O. The deprotonated amide functions as both σ - and π -donors, as observed in previous calculations for a wild-type Ni^{2+} SOD model [43]. To lower energy, the occupied MOs of both C2S/C6S^{5-c} and C2S/C6S^{6-c} possess primarily Ni $d(xy)$ (MOs 72 and 78), Ni $d(xz)$ (MOs 74 and 79), and Ni $d(yz)$ (MOs 73 and 77) orbital character. As anticipated, removal of the axial water molecule from C2S/C6S^{6-c} to generate the C2S/C6S^{5-c} model leads to a significant stabilization of the Ni $d(z^2)$ -based MO (by 0.36 eV) owing to the elimination of the σ -antibonding interaction between the Ni $d(z^2)$ and O_{H2O} $p_{x,y}$ orbitals. The reduction in coordination number and consequent increase in the N(His1)–Ni–N and N(His)–Ni–O bond angles also slightly affects the energies of the remaining MOs, causing a minor stabilization of the empty Ni $d(x^2 - y^2)$ -based and filled Ni *d*-based spin-down MOs.

Replacing the anionic Cys residues in wild-type Ni-SOD_{red} [43] with neutral Ser residues to generate the C2S/C6S^{5-c} and C2S/C6S^{6-c} active-site models results in a considerable stabilization of the Ni *d*-based MOs relative to the ligand-based MOs, as well as a

significant change in the compositions of the Ni *d*-based MOs. For example, whereas the unoccupied Ni $d(x^2 - y^2)$ -based MO of the wild-type ($S = 0$) Ni²⁺SOD active-site model contains approximately 43% Ni *d* and 21% S_{Cys2,Cys6} orbital character (spin-restricted calculation) [43], the Ni $d(x^2 - y^2)$ -based spin-down MOs for both C2S/C6S NiSOD models have approximately 75% Ni *d* and only about 4% O_{Ser2,Ser6} orbital character.

To assess which of the two C2S/C6S NiSOD models provides a better description of the mutant's active site, the TD-DFT-computed transition energies and electronic absorption intensities for the two models were compared with the corresponding experimental data (Fig. 10). For both the C2S/C6S^{6-c} model and the C2S/C6S^{5-c} model, LF transitions are predicted to be responsible for bands 1–4, 8, and 9, in agreement with the experimental data for C2S/C6S NiSOD. According to our spin-unrestricted TD-DFT calculations for both the C2S/C6S^{6-c} model and the C2S/C6S^{5-c} model, one-electron excitations from the filled Ni $d(xz)$ -based and Ni $d(yz)$ -based spin-down MOs to the unoccupied Ni $d(z^2)$ -based spin-down MO give rise to the lowest energy MCD features (bands 1 and 2), whereas the highest energy features (bands 8 and 9) derive from the Ni $d(xz) \rightarrow$ and Ni $d(yz) \rightarrow d(x^2 - y^2)$ transitions. Although these assignments are qualitatively consistent with our spectral deconvolution data (vide supra), the TD-DFT-computed electronic absorption transition energies and intensities for the two models are very different. The overall calculated electronic absorption intensity increases by approximately threefold from the six-coordinate, C2S/C6S^{6-c} model to the five-coordinate, C2S/C6S^{5-c} model. A systematic red shift of the LF transitions is also observed with reduction of the coordination number in the active-site models. Similar trends were noted from experimental studies of analogous five- and six-coordinate ($S = 1$) Ni²⁺ complexes upon partial or complete dissociation of an axial ligand [84]. From our DFT computations, these shifts are due, primarily, to a stabilization of the Ni $d(z^2)$ -derived and Ni $d(x^2 - y^2)$ -derived MOs from C2S/C6S^{6-c} to C2S/C6S^{5-c}.

For both C2S/C6S NiSOD active-site models, the energy of the Ni $d(yz) \rightarrow d(x^2 - y^2)$ transition (band 9) is underestimated by approximately 7,000 cm⁻¹. This is not unexpected, though, given that for the H134A urease active-site model the corresponding transition energy is similarly underestimated by the TD-DFT method. Comparison of the computed and experimental energies of the remaining transitions (Fig. 10) reveals that the C2S/C6S^{6-c} model yields considerably better agreement than the C2S/C6S^{5-c} model, especially with regard to the position of band 1. Hence, our TD-DFT results lend further support to the proposal that the Cys $\rightarrow$ Ser NiSOD mutants possess six-coordinate active sites, as initially inferred from our MCD spectral data. Our spectroscopic and computational data additionally suggest that a solvent molecule coordinates to the Ni²⁺ ion of the Cys $\rightarrow$ Ser NiSOD mutants, to complete a distorted O_h coordination sphere.

Discussion

NiSOD is unique among the family of SODs, as its active site features an N-terminal amine, a deprotonated amide, and *cis*-cysteineate ligands [24,25]. Coordination of the latter residues to the Ni center is particularly unusual in the context of SOD catalysis, as thiolates are readily modified by strong oxidants [32–42]. However, Cys coordination in NiSOD is consistent with the notion that Ni-containing metalloenzymes featuring Ni–thiolate interactions are redox-active, whereas Ni-containing proteins with Ni–N/O coordination spheres are redox-inactive [46]. In [63], the necessity for the Cys residues with respect to the NiSOD activity was demonstrated by constructing a series of Cys $\rightarrow$ Ser NiSOD mutants. Alteration of one (C2S and C6S NiSOD) or both Cys residues (C2S/C6S NiSOD) led to access to the Ni³⁺ state being hindered, indicating an increase in the Ni^{3+/2+} potential [63]. Additionally, the Cys $\rightarrow$ Ser mutations induced changes in both the geometric and the electronic structures of the active sites, which was particularly evident from the C2S, C6S,

and C2S/C6S NiSOD EPR and EXAFS data [63]. In this study, we employed various spectroscopic and computational tools to further characterize the active sites of the Cys → Ser NiSOD mutants. Key insights from our studies are summarized below, and possible implications for the lack of catalytic activity displayed by these mutants are discussed.

EPR studies of the Cys → Ser NiSOD mutants established that the active site contains exclusively Ni²⁺ [63]. However, discrimination between a low-spin ($S = 0$) or a high-spin ($S = 1$) electronic configuration for the Ni²⁺ center was not possible because high-spin Ni²⁺ systems are typically subject to a large zero-field splitting, and are thus “EPR-silent” when using X-band microwave frequencies [89]. To address this issue, we employed MCD spectroscopy, which is uniquely sensitive to paramagnetic centers [88]. The temperature dependence of the mutants’ MCD features undoubtedly reveals that each mutant contains a high-spin Ni²⁺ ion. The lack of any significant differences among the electronic absorption, CD, MCD, and EXAFS [63] data of the C2S, C6S, and C2S/C6S NiSOD mutants demonstrates that all three species possess very similar active-site geometric and electronic structures. This finding strongly suggests that the remaining Cys residue in the single mutants is not coordinated to the metal center, but is instead replaced by an O-donor ligand, resulting in an active site similar to that of C2S/C6S NiSOD. Hence, it appears that mutation of one Cys residue to a Ser in C2S and C6S NiSOD drastically weakens the bonding interaction between the Ni d orbitals and the Cys S-based orbitals, thereby preventing coordination of the remaining Cys to the Ni²⁺ ion. This hypothesis is supported by the fact that high-spin, O_h Ni²⁺ centers with N/O/S [121] and N/S [122–124] coordination spheres exhibit rather large Ni–thiolate bond distances of approximately 2.4 Å, as compared with the distance of about 2.2 Å for analogous square-planar ($S = 0$) Ni²⁺ systems [33,58,125,126]. Therefore, it seems that the inability of Cys to coordinate the Ni center in C2S and C6S NiSOD is partially intrinsic to the high-spin configuration of the Ni²⁺ ion, and is partially due to the unique ligand environment imposed by the enzyme. In the synthetic complexes, the difference in Ni–S distances for $S = 0$ and $S = 1$ Ni²⁺ species is readily understood in terms of the different ground-state electron configurations. Square-planar ($S = 0$) Ni²⁺ complexes generally possess unoccupied Ni $d(x^2 - y^2)$ -based MOs, whereas octahedral ($S = 1$) Ni²⁺ complexes have singly occupied Ni $d(x^2 - y^2)$ -based and Ni $d(z^2)$ -based MOs [87]. Occupation of the Ni²⁺ $d(x^2 - y^2)$ -based orbital lowers the Ni–S bond order and, thus causes a significant lengthening of these bonds in octahedral complexes.

Ni²⁺ ligand environment of the Cys → Ser NiSOD mutants

On the basis of the close resemblance of the MCD spectra of the Cys → Ser NiSOD mutants and those of a variety of high-spin ($S = 1$) Ni²⁺ species featuring distorted six-coordinate ligand environments [95–103], specifically the Ni²⁺ active site of H134A urease [64], a similar active-site coordination for the mutants can be inferred. The main MCD features are therefore attributed to the spin-allowed transitions from the $^3A_{2g}$ ground state to the $^3T_{2g}$, $^3T_{1g}(F)$, and $^3T_{1g}(P)$ excited states (in the parent O_h symmetry). In agreement with these assignments, the band positions are comparable to those reported for octahedral Ni²⁺ systems [85,87], and the TD-DFT computations for the C2S/C6S NiSOD active-site models predict that the discernable MCD features arise from LF transitions terminating in the Ni $d(z^2)$ -based and Ni $d(x^2 - y^2)$ -based spin-down MOs. The intensities of the major MCD features are also characteristic of a six-coordinate Ni²⁺ ligand environment, as five-coordinate Ni²⁺ species typically exhibit considerably more intense MCD features [99,103].

Additional support for the proposed six-coordinate, distorted O_h ligand environment of the Ni²⁺ centers in the Cys → Ser NiSOD mutants’ active-site sphere was obtained by collecting MCD data at low pH. For each mutant, sizeable red shifts were observed for bands 1, 2, and 4 upon lowering the pH from 8.5 to 5.5, consistent with a weakening of an axial ligand–Ni²⁺ bonding interaction. Given the presence of two ordered water molecules

near the vacant axial position of the Ni center in the wild-type NiSOD_{ox} and NiSOD_{red} crystal structures [24,25], these results likely imply that a solvent molecule is axially coordinated to the high-spin Ni²⁺ ion of each Cys → Ser NiSOD mutant. Because the observed red shifts are smaller than expected for the conversion of an H₂O ligand at low pH to an OH⁻ ligand at high pH, the pK_a of the coordinated solvent presumably lies outside the range of pH values investigated (presumably greater than 8.5 pH units). A similar tendency for a high-spin Ni²⁺ center to expand its coordination sphere via coordination of a solvent molecule has been reported for the catalytically inactive, Ni²⁺-substituted Zn²⁺ endopeptidase [127,128].

Although our MCD and computational data strongly favor a six-coordinate, distorted O_h Ni²⁺ ligand environment for the Cys → Ser NiSOD mutants, the electronic absorption and XAS data obtained for these species appear to be more consistent with a lower coordination number. In particular, the Ni K-edge region, commonly used to probe the active-site coordination geometry of Ni-containing proteins and enzymes [64,129–132], was found to exhibit peak areas of the features associated with the Ni 1s → 3d transitions that are characteristic of a five-coordinate Ni²⁺ active-site [63]. Moreover, a relatively intense Ni 1s → 4p(z) feature was observed in the XAS spectra of all three Cys → Ser NiSOD mutants, signifying a significant deviation from inversion symmetry of the Ni²⁺ center [132]. To reconcile these XAS data with our MCD and computational results, the effect of a partial dissociation of the coordinated solvent ligand on the spectroscopic properties of the Cys → Ser NiSOD mutants was analyzed computationally by systematically varying the Ni–OH₂ bond distance of the C2S/C6S^{6-c} model and using TD-DFT to compute the electronic absorption spectrum for each of the distorted models (Fig. 11, Table 4). These computations suggest that the LF transitions steadily shift to lower energy when the Ni–OH₂ bond length is increased from its equilibrium value of 2.57 Å. For a Ni–OH₂ bond elongation of 0.45 Å, transitions terminating in the Ni d(z²)-based MO are predicted to exhibit red shifts of up to 1,200 cm⁻¹ owing to the large stabilization of this orbital in response to a weakening of the Ni–OH₂ σ-antibonding interaction. Alternatively, transitions terminating in the Ni d(x² – y²)-based MO are expected to display only minor red shifts, because the energy of this orbital is fairly insensitive to changes in the Ni–OH₂ bond length.

A comparison of the TD-DFT-computed electronic absorption spectra for C2S/C6S^{6-c} and the distorted active-site model with a Ni–OH₂ bond length of 3.02 Å reveals significant differences in terms of their transition energies and intensities (Fig. 11, Table 4). In particular, the overall absorption intensity is substantially higher for the perturbed C2S/C6S^{6-c} model than for the geometry-optimized C2S/C6S^{6-c} model, and a substantial red-shift is predicted for band 1 upon elongation of the Ni–OH₂ bond. Nonetheless, the computed electronic absorption spectra for these two models are qualitatively remarkably similar to one another (Fig. 11). Therefore, the electronic structure of the perturbed C2S/C6S^{6-c} model (Ni–OH₂ bond length of 3.02 Å) is in many ways reminiscent of that of a genuine six-coordinate Ni²⁺ complex, even though its geometric structure is more consistent with a five-coordinate Ni²⁺ species. On the basis of this analysis, we propose that the Ni–OH₂ bond distance in the Cys → Ser NiSOD mutants is unusually large, which would explain why seemingly contradictory conclusions were drawn with regard to the coordination number of the Ni²⁺ ion from our MCD and DFT results on the one hand and the XAS data obtained by Ryan et al. [63] on the other.

Factors contributing to catalytic inactivity of the Cys → Ser NiSOD mutants

An evaluation of the two C2S/C6S NiSOD active-site models considered in light of our CD, MCD, and TD-DFT data strongly favors the six-coordinate model (C2S/C6S^{6-c}) over the five-coordinate model (C2S/C6S^{5-c}). Therefore, the electronic structure of C2S/C6S^{6-c} is used here to explore why the replacement of one or both active-site Cys residues by Ser

completely abolishes the catalytic activity of NiSOD (note that the same basic conclusions would be reached by considering the electronic structure of the C2S/C6S^{5-c} model).

The bonding description for C2S/C6S^{6-c} indicates that the frontier MOs are largely composed of Ni 3*d* and amide N/O-based orbitals. The lack of any orbital contributions from the neutral Ser residues is a direct reflection of the low σ - and π -donor strengths of these ligands. In contrast, in wild-type NiSOD_{red} the Cys residues engage in highly covalent Ni–S bonding interactions, as evidenced by the large $S(\sigma)$ and $S(\pi)$ contributions to the corresponding frontier MOs [43]. It is, therefore, not surprising that the Cys → Ser NiSOD mutants exhibit a strong preference for a high-spin Ni²⁺ configuration, while a diamagnetic Ni²⁺ center is observed for NiSOD_{red}. Recent computational work on a NiSOD_{red} active-site model revealed that the interactions between the Cys $S(\pi)$ and Ni $d(\pi)$ -type orbitals in the HOMO and HOMO-1 are essential for the reduction of O₂^{•-} and the stability of NiSOD_{ox} [43]. Similarly strong Cys $S(\pi)$ /Ni $d(\pi)$ bonding interactions were found to exist in the reduced state of NiSOD mimics [37,133]. We therefore conclude that a major factor contributing to the lack of catalytic activity displayed by the Cys → Ser NiSOD mutants is the destabilization of the Ni³⁺ state. In fact, our spectroscopic and computational data provide compelling evidence that both active-site Cys residues are needed to lower the Ni^{3+/2+} potential enough to allow access to the Ni³⁺ state and, thus, to equip wild-type NiSOD with the ability to catalyze the reductive half-reaction in the catalytic cycle of O₂^{•-} disproportionation (Eq. 2). In strong support of this proposal, electrochemical studies of square-planar, low-spin ($S = 0$) Ni²⁺ thiolate complexes [57,58,134] revealed that a chemical modification of the thiolates by alkylation [40,41] or oxygenation [33,36,37] significantly increases the Ni^{3+/2+} potential and even provides access to the Ni^{2+/+} redox couple [135]. Additionally, considering the proposed coordination of neutral Ser residues in the C2S/C6S NiSOD active site, it is conceivable that imposing a positive charge on the active site would raise the Ni^{3+/2+} potential even further and thus inhibit access to the Ni³⁺ state. Based on the similar geometric and electronic structures of all three Cys → Ser NiSOD mutants investigated, a similar conclusion can be drawn for the inactivity of the C2S and C6S NiSOD variants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (Grant GM 64631 to T.C.B.), the University of Wisconsin Chemical Biology Interface Training Grant from the National Institutes of Health (Grant T32 GM008505 to O.E.J.), and the National Science Foundation (Grant CHE-0809188 to M.J.M.).

Abbreviations

B3LYP	Becke's three-parameter hybrid functional for exchange combined with the Lee–Yang–Par correlation functional
CD	Circular dichroism
Cys → Ser	Cysteine to serine
DFT	Density functional theory
EPR	Electron paramagnetic resonance
EXAFS	Extended X-ray absorption fine structure

HOMO	Highest occupied molecular orbital
INDO/S-CI	Intermediate neglect of differential overlap/spectroscopic parameterization with configuration interaction
LF	Ligand field
MCD	Magnetic circular dichroism
MO	Molecular orbital
NiSOD	Nickel-dependent superoxide dismutase
NiSOD_{ox}	Oxidized nickel-dependent superoxide dismutase
NiSOD_{red}	Reduced nickel-dependent superoxide dismutase
SOD	Superoxide dismutase
TD-DFT	Time-dependent density functional theory
Tris	Tris(hydroxymethyl)aminomethane

References

1. Imlay JA. *Annu Rev Microbiol* 2003;57:395–418. [PubMed: 14527285]
2. Midorikawa K, Kawanishi S. *FEBS Lett* 2001;495:187–190. [PubMed: 11334889]
3. Valentine JS, Wertz DL, Lyons TJ, Liou L-L, Goto JJ, Gralla EB. *Curr Opin Chem Biol* 1998;2:253–262. [PubMed: 9667937]
4. Moller IM. *Annu Rev Plant Physiol Plant Mol Biol* 2001;52:561–591. [PubMed: 11337409]
5. Simonian NA, Coyle JT. *Rev Pharmacol Toxicol* 1996;36:83–106.
6. Beyer W, Imlay J, Fridovich I. *Prog Nucl Acid Res Mol Biol* 1991;40:221–253.
7. Brown RHJ. *Cold Spring Harb Monogr Ser* 1997;34:569–585.
8. Chatterjee, S.; Fisher, AB. *Phytopharmaceuticals in cancer chemoprevention*. Bagchi, D.; Preuss, HG., editors. CRC Press; Boca Raton: 2005. p. 171-186.
9. Halliwell B. *Methods Enzymol* 1990;186:1–85. [PubMed: 2172697]
10. Halliwell, B. *Active oxygen in biochemistry*. Valentine, JS.; Foote, CS.; Greenberg, A.; Liebman, JF., editors. Blackie; New York: 1995. p. 313-335.
11. Jenner P, Olanow CW. *Ann Neurol* 1998;44:S72–S84. [PubMed: 9749577]
12. Passi S, Ricci R, Aleo E, Cocchi M. *Prog Nutr* 2005;7:3–22.
13. Trotti D, Rolfs A, Danbolt NC, Brown JRH, Hediger MA. *Nat Neurosci* 1999;2:427–433. [PubMed: 10321246]
14. Wallace DC. *Science* 1992;256:628–632. [PubMed: 1533953]
15. Valentine JS, Hart PJ. *Proc Natl Acad Sci USA* 2003;100:3617–3622. [PubMed: 12655070]
16. Kurtz DM. *Acc Chem Res* 2004;37:902–908. [PubMed: 15612680]
17. Miller AF. *Curr Opin Chem Biol* 2004;8:162–168. [PubMed: 15062777]
18. Miller AF, Sorkin DL. *Commun Mol Cell Biophys* 1997;9:1–48.
19. Fridovich I. *Annu Rev Biochem* 1995;64:97–112. [PubMed: 7574505]
20. Fridovich, I.; William, JL.; Lane, MD. *Encyclopedia of biological chemistry*. Elsevier; New York: 2004. p. 135-138.
21. Youn HD, Kim EJ, Roe JH, Hah YC, Kang SO. *Biochem J* 1996;318:889–896. [PubMed: 8836134]
22. Youn HD, Youn H, Lee JW, Yim YI, Lee JK, Hah YC, Kang SO. *Arch Biochem Biophys* 1996;334:341–348. [PubMed: 8900409]

23. Palenik B, Brahamsha B, Larimer FW, Land M, Hauser L, Chain P, Lamerdin J, Regala W, Allen EE, McCarren J, Paulsen I, Dufresne A, Partensky F, Webb EA, Waterbury J. *Nature* 2003;424:1037–1042. [PubMed: 12917641]
24. Barondeau DP, Kassmann CJ, Bruns CK, Tainer JA, Getzoff ED. *Biochemistry* 2004;43:8038–8047. [PubMed: 15209499]
25. Wuerges J, Lee JW, Yim YI, Yim HS, Kang SO, Carugo KD. *Proc Natl Acad Sci USA* 2004;101:8569–8574. [PubMed: 15173586]
26. Hart PJ, Balbirnie MM, Ogihara NL, Nersissian AM, Weiss MS, Valentine JS, Eisenberg D. *Biochemistry* 1999;38:2167–2178. [PubMed: 10026301]
27. Doukov TI, Iverson TM, Seravalli J, Ragsdale SW, Drennan CL. *Science* 2002;298:567–572. [PubMed: 12386327]
28. Kovacs JA. *Chem Rev* 2004;104:825–848. [PubMed: 14871143]
29. Chan Chung KC, Cao L, Dias AV, Pickering IJ, George GN, Zamble DB. *J Am Chem Soc* 2008;130:14056–14057. [PubMed: 18834129]
30. Harford C, Sarkar B. *Acc Chem Res* 1997;30:123–130.
31. Lanzilotta WN, Schuller DJ, Thorsteinsson MV, Kerby RL, Roberts GP, Poulos TL. *Nat Struct Biol* 2000;7:876–880. [PubMed: 11017196]
32. Chohan BS, Maroney MJ. *Inorg Chem* 2006;45:1906–1908. [PubMed: 16499349]
33. Farmer PJ, Reibenspies JH, Lindahl PA, Darensbourg MY. *J Am Chem Soc* 1993;115:4665–4674.
34. Farmer PJ, Solouki T, Mills DK, Soma T, Russell DH, Reibenspies JH, Darensbourg MY. *J Am Chem Soc* 1992;114:4601–4605.
35. Grapperhaus CA, Darensbourg MY. *Acc Chem Res* 1998;31:451–459.
36. Grapperhaus CA, Mullins CS, Kozlowski PM, Mashuta MS. *Inorg Chem* 2004;43:2859–2866. [PubMed: 15106973]
37. Green KN, Brothers SM, Jenkins RM, Carson CE, Grapperhaus CA, Darensbourg MY. *Inorg Chem* 2007;46:7536–7544. [PubMed: 17685511]
38. Kaasjager VE, Bouwman E, Gorter S, Reedijk J, Grapperhaus CA, Reibenspies JH, Smee JJ, Darensbourg MY, Derecskei-Kovacs A, Thomson LM. *Inorg Chem* 2002;41:1837–1844. [PubMed: 11925177]
39. Kumar M, Colpas GJ, Day RO, Maroney MJ. *J Am Chem Soc* 1989;111:8323–8325.
40. Bellefeuille JA, Grapperhaus CA, Derecskei-Kovacs A, Reibenspies JH, Darensbourg MY. *Inorg Chim Acta* 2000;300–302:73–81.
41. Smee JJ, Miller ML, Grapperhaus CA, Reibenspies JH, Darensbourg MY. *Inorg Chem* 2001;40:3601–3605. [PubMed: 11421712]
42. Allan CB, Davidson G, Choudhury SB, Gu Z, Bose K, Day RO, Maroney MJ. *Inorg Chem* 1998;37:4166–4167. [PubMed: 11670546]
43. Fiedler AT, Bryngelson PA, Maroney MJ, Brunold TC. *J Am Chem Soc* 2005;127:5449–5462. [PubMed: 15826182]
44. Neupane KP, Shearer J. *Inorg Chem* 2006;45:10552–10566. [PubMed: 17173410]
45. Shearer J, Dehestani A, Abanda F. *Inorg Chem* 2008;47:2649–2660. [PubMed: 18330983]
46. Maroney MJ. *Curr Opin Chem Biol* 1999;3:188–199. [PubMed: 10226043]
47. Volbeda A. *Nature* 1995;373:580–587. [PubMed: 7854413]
48. Dobbek H, Svetlitchnyi V, Gremer L, Huber R, Meyer O. *Science* 2001;293:1281–1285. [PubMed: 11509720]
49. Ermler U, Grabarse W, Shima S, Goubeaud M, Thauer RK. *Science* 1997;278:1457–1462. [PubMed: 9367957]
50. Heddle J, Scott DJ, Unzai S, Park S-Y, Tame JRH. *J Biol Chem* 2003;278:50322–50329. [PubMed: 12960164]
51. Jabri E, Carr MB, Hausinger RP, Karplus PA. *Science* 1995;268:998–1004. [PubMed: 7754395]
52. Song HK, Mulrooney SB, Huber R, Hausinger RP. *J Biol Chem* 2001;276:49359–49364. [PubMed: 11591723]
53. Watt RK, Ludden PW. *Cell Mol Life Sci* 1999;56:604–625. [PubMed: 11212309]

54. Choudhury SB, Lee JW, Davidson G, Yim YI, Bose K, Sharma ML, Kang SO, Cabelli DE, Maroney MJ. *Biochemistry* 1999;38:3744–3752. [PubMed: 10090763]
55. Herbst RW, Guce A, Bryngelson PA, Higgins KA, Rynan KC, Cabelli DE, Garman SC, Maroney MJ. *Biochemistry* 2009;48:3354–3369. [PubMed: 19183068]
56. Barrette WC, Sawyer DT, Fee JA, Asada K. *Biochemistry* 1983;22:624–627. [PubMed: 6340720]
57. Krueger HJ, Holm RH. *Inorg Chem* 1987;26:3645–3647.
58. Krueger HJ, Peng G, Holm RH. *Inorg Chem* 1991;30:734–742.
59. Ray D, Pal S, Chakravorty A. *Inorg Chem* 1986;25:2674–2677.
60. Jackson TA, Brunold TC. *Acc Chem Res* 2004;37:461–470. [PubMed: 15260508]
61. Xie J, Yikilmaz E, Miller AF, Brunold TC. *J Am Chem Soc* 2002;124:3769–3774. [PubMed: 11929267]
62. Tietze D, Breitke H, Imhof D, Kothe E, Weston J, Buntkowsky G. *Chem Eur J* 2009;15:517–523.
63. Ryan KC, Johnson OE, Brunold TC, Johnson MK. *J Biol Inorg Chem*. 201010.1007/s00775-010-0645-y
64. Park IS, Michel LO, Pearson MA, Jabri E, Karplus PA, Wang SK, Dong J, Scott RA, Koehler BP, Johnson MK, Hausinger RP. *J Biol Chem* 1996;271:18632–18637. [PubMed: 8702515]
65. Baerends EJ, Ellis DE, Ros P. *Chem Phys* 1973;2:41–51.
66. Guerra CF, Snijders JG, te Velde G, Baerends EJ. *Theor Chem Acc* 1998;99:391–403.
67. te Velde G, Baerends EJ. *J Comput Phys* 1992;99:84–98.
68. te Velde G, Bickelhaupt FF, Baerends EJ, Guerra CF, Van Gisbergen SJA, Snijders JG, Ziegler T. *J Comput Chem* 2001;22:931–967.
69. Versluis L, Ziegler T. *J Chem Phys* 1988;88:322–328.
70. Vosko SH, Wilk L, Nusair M. *Can J Phys* 1980;58:1200.
71. Becke AD. *J Chem Phys* 1993;98:5648–5652.
72. Perdew JP. *Phys Rev B* 1986;33:8822–8824.
73. Neese, F. ORCA 2.4. Max-Planck-Institut für Bioanorganische Chemie; Mülheim an der Ruhr: 2005.
74. Ridley J, Zerner MC. *Theor Chim Acta* 1973;32:111.
75. Zerner MC, Loew GH, Kirchner RF, Muellerwesterhoff UT. *J Am Chem Soc* 1980;102:589–599.
76. Bacon AD, Zerner MC. *Theor Chim Acta* 1979;53:21–54.
77. Becke AD. *J Chem Phys* 1993;98:1372–1377.
78. Lee C, Yang W, Parr RG. *Phys Rev B* 1988;37.
79. Schafer A, Horn H, Ahlrichs R. *J Chem Phys* 1992;97:2571–2577.
80. Weigand F, Haeser M. *Theor Chem Acc* 1997;97:331.
81. Schafer A, Huber C, Ahlrichs R. *J Chem Phys* 1994;100:5829–5835.
82. Laaksonen L. *J Mol Graph* 1992;10:33–34. [PubMed: 1504051]
83. Boge EM, Freyberg DP, Kokot E, Mockler GM, Sinn E. *Inorg Chem* 1977;16:1655–1660.
84. Hammes BS, Carrano CJ. *Inorg Chem* 1999;38:3562–3568. [PubMed: 11671105]
85. Martin LY, Sperati CR, Busch DH. *J Am Chem Soc* 1977;99:2968–2981.
86. Cameron JH, McCullough KJ. *J Chem Soc Dalton Trans* 1990:935–941.
87. Lever, ABP. *Inorganic Electronic Spectroscopy*. Elsevier; New York: 1984. p. 507-530.
88. Johnson, MK. *Physical methods in bioinorganic chemistry: spectroscopy and magnetism*. Que, L., Jr, editor. University Science Books; Sausalito: 2000. p. 253-259.
89. Desrochers PJ, Telser J, Zvyagin SA, Ozarowski A, Krzystek J, Vicic DA. *Inorg Chem* 2006;45:8930–8941. [PubMed: 17054352]
90. Steenkamp PJ. *Inorg Chim Acta* 1987;132:27–31.
91. Kolis JW, Hamilton DE, Kildahl NK. *Inorg Chem* 1979;18:1826–1831.
92. Sacconi L, Ciampolini M, Speroni GP. *J Am Chem Soc* 1965;87:3102–3106.
93. Sacconi L, Nannelli P, Nardi N, Campigli U. *Inorg Chem* 1965;4:943–949.
94. Sacconi L, Orioli PL, Di Vaira M. *Inorg Chem* 1996;4:943–949.

95. Arakawa S, Nozawa T, Hatano M. *Bull Chem Soc Jpn* 1974;47:2643–2646.
96. Harding MJ, Mason SF, Robbins DJ, Thomson AJ. *J Chem Soc A* 1971;19:3058–3062.
97. Harding MJ, Mason SF, Robbins DJ, Thomson AJ. *J Chem Soc A* 1971;19:3047–3058.
98. Hormann E, Riesen PC, Neuburger M, Zehnder M, Kaden TA. *Helv Chim Acta* 1996;79:235–243.
99. Katô H, Yorita K, Kato Y. *Bull Chem Soc Jpn* 1979;52:2465–2473.
100. Kobayashi H, Bohdan K-D. *Bull Chem Soc Jpn* 1972;45:2485–2490.
101. Kobayashi N, Xizhang Z, Tetsuo O. *J Chem Soc Dalton Trans* 1987:1801–1803.
102. Scheiner AF, Hamm DJ. *Inorg Chem* 1973;12:2037–2048.
103. Kiick, KL. *Chemistry*. University of Georgia; Athens: 1991. p. 180
104. Averill DF, Legg JI, Smith DL. *Inorg Chem* 1971;11:2344–2349.
105. Fukuda Y, Yamagata K, Kaden TA. *Bull Chem Soc Jpn* 1987;60:3569–3574.
106. Ohtsu H, Tanaka K. *Inorg Chem* 2003;43:3024–3030. [PubMed: 15106994]
107. Ohtsu H, Tanaka K. *Chem Eur J* 2005;11:3420–3426.
108. Neupane KP, Gearty K, Francis A, Shearer J. *J Am Chem Soc* 2007;129:14605–14618. [PubMed: 17985883]
109. Battaglia LP, Berzolla PG, Corradi AB, Pelizzi C, Pelosi G, Solinas C. *J Chem Soc Dalton Trans* 1992:3089–3095.
110. Dobrzynska D, Jerzykiewicz LB, Duczmal M, Wojciechowska A, Jablonska K, Palus J, Ozarowski A. *Inorg Chem* 2006;45:10479–10486. [PubMed: 17173403]
111. Du Q-Y, Li Y-P, Xin L-Y, Han M-L. *Z Kristallogr NCS* 2005;220:539–540.
112. Ihara Y, Satake Y, Fujimoto Y, Senda H, Suzuki M, Uehara A. *Bull Chem Soc Jpn* 1991;64:2349–2352.
113. Ide DMM, Norman RE. *Acta Crystallogr E* 2007;63:m558–m560.
114. Atkinson IM, Byriel KA, Chia PSK, Kennard CHL, Leong AJ, Lindoy LF, Lowe MP, Mahendran S, Smith G, Wei G. *Aust J Chem* 1998;51:985–991.
115. Thulstrup PN, Broge L, Larsen E, Springborg J. *J Chem Soc Dalton Trans* 2003:3199–3204.
116. Xiao Z, Lavery MJ, Ayhan M, Scrofanì SDB, Wilce MCJ, Guss JM, Tregloan PA, George GN, Wedd AG. *J Am Chem Soc* 1998;120:4135–4150.
117. Pons J, Chadghan A, Casabo J, Alvarez-Larena A, Piniella JF, Solans X, Font-Bardia M, Ros J. *Polyhedron* 2001;20:1029–1035.
118. Elernson Y, Kabak M. *Acta Crystallogr C* 1993;49:1905–1906.
119. Nielson DO, Larsen ML, Willet RD, Legg JI. *J Am Chem Soc* 1971;93:5079–5082.
120. Shintani N, Sone K, Fukuda Y, Ohashi Y. *Bull Chem Soc Jpn* 1991;64:252–258.
121. Calatayud ML, Castro I, Sletten J, Cano J, Lloret F, Faus J, Julve M, Seitz G, Mann K. *Inorg Chem* 1996;35:2858–2865.
122. Stranger R, McMahon KL, Gahan LR, Bruce JI, Hambley TW. *Inorg Chem* 1997;36:3466–3475. [PubMed: 11670023]
123. Cha M, Gatlin CL, Critchlow SC, Kovacs JA. *Inorg Chem* 2002;32:5868–5877.
124. Chohan BS, Shoner SC, Kovacs JA, Maroney MJ. *Inorg Chem* 2004;43:7726–7734. [PubMed: 15554637]
125. Gomes L, Pereira E, de Castro B. *J Chem Soc Dalton Trans* 2000:1373–1379.
126. Dutton JC, Fallon GD, Murray KS. *Chem Lett* 1990:983–986.
127. Lipscomb WN, Strater N. *Chem Rev* 1996;96:2375–2434. [PubMed: 11848831]
128. Ruth-Gomis F-X, Grams F, Yiallouris I, Nar H, Kusthardt U, Zwillig R, Bode W, Stocker W. *J Biol Chem* 1994;269:17111–17117. [PubMed: 8006015]
129. Lee MH, Pankratz HS, Wang S, Scott RA, Finnegan MG, Johnson MK, Ippolito JA, Christianson DW, Hausinger RP. *Protein Sci* 1993;2:1042–1052. [PubMed: 8318889]
130. Carrington PE, Chivers PT, Al-Mjeni F, Sauer RT, Maroney MJ. *Nat Struct Biol* 2003;10:126–130. [PubMed: 12524532]
131. Davidson G, Clugston SL, Honek JF, Maroney MJ. *Inorg Chem* 2000;39:2962–2963. [PubMed: 11196887]

132. Haumann M, Porthun A, Buhrke T, Liebisch P, Meyer-Klaucke W, Friedrich B, Dau H. *Biochemistry* 2003;42:11004–11015. [PubMed: 12974636]
133. Mullins CS, Grapperhaus CA, Kozlowski PM. *J Biol Chem* 2006;11:617–625.
134. Fox S, Wang Y, Silver A, Millar M. *J Am Chem Soc* 1990;112:3218–3220.
135. Dey A, Jeffery SP, Darensbourg M, Hodgson KO, Hedman B, Solomon EI. *Inorg Chem* 2007;46:4989–4996. [PubMed: 17500514]

Fig. 1. Truncated structures of the wild-type Ni-dependent superoxide dismutase (NiSOD) active site in the oxidized (*NiSOD_{ox}*) and reduced (*NiSOD_{red}*) states

Fig. 2. Room temperature electronic absorption spectra of C2S (*dotted line*), C6S (*broken line*), and C2S/C6S (*solid line*) NiSOD at pH 8.5

Fig. 3. Variable-temperature magnetic circular dichroism (MCD) spectra collected at 7 T and 4, 8, 15, 30, and 50 K of C2S (*top*), C6S (*middle*), and C2S/C6S (*bottom*) NiSOD at pH 8.5

Fig. 4.

Gaussian deconvolution of the C2S/C6S NiSOD electronic absorption (298 K), circular dichroism (CD) (298 K), and MCD (4 K, 7 T) spectra at pH 8.5. Experimental (*solid line*), individual Gaussian bands (*dotted line*), and Gaussian fit (*broken line*)

Fig. 5. MCD spectra at 4 K/7 T of C2S (*top*), C6S (*middle*), and C2S/C6S (*bottom*) NiSOD at pH 8.5 (*solid line*) and pH 5.5 (*broken line*). The bands most strongly affected by a change in pH are highlighted by an *asterisk*

Fig. 6. Room temperature (*solid line*) and 4 K (*broken line*) CD spectra of C2S (*top*), C6S (*middle*), and C2S/C6S (*bottom*) NiSOD at pH 8.5

Fig. 7. MCD spectra at 4 K/7 T of resting (*solid line*) and azide-treated (*broken line*) C2S (*top*), C6S (*middle*), and C2S/C6S (*bottom*) NiSOD at pH 8.5

Fig. 8.
Density functional theory (DFT)-optimized active-site model of H134A urease

Fig. 9. Energies and isosurface plots of the relevant spin-down molecular orbitals of the C2S/C6S^{6-c} (*left*) and C2S/C6S^{5-c} (*right*) NiSOD active-site models as obtained from spin-unrestricted density functional theory (DFT) calculations

Fig. 10.

Experimental electronic absorption spectrum of C2S/C6S NiSOD (*top*) and the time-dependent DFT (TD-DFT)-computed electronic absorption spectra for the C2S/C6S^{5-c} (*middle*) and C2S/C6S^{6-c} (*bottom*) NiSOD active-site models

Fig. 11.

TD-DFT-computed electronic absorption spectra for a series of C2S/C6S^{6-c} active-site models with differing Ni-OH₂ bond lengths (as indicated on the *left*). The unperturbed model has a Ni-OH₂ bond length of 2.57 Å. The TD-DFT-computed electronic absorption spectrum for the C2S/C6S^{5-c} active-site model is included at the *bottom* for comparison

Scheme 1.

DFT-optimized active-site models of C2S/C6S NiSOD possessing six-coordinate (C2S/C6S^{6-c}) and five-coordinate (C2S/C6S^{5-c}) Ni²⁺ environments

Table 1

Electronic transition energies and extinction coefficients derived from the Gaussian analysis of the C2S/C6S Ni-dependent superoxide dismutase (NiSOD) electronic absorption (*Abs*), circular dichroism (*CD*), and magnetic circular dichroism (*MCD*) spectra at pH 8.5

Band	Energy ^a (cm ⁻¹)	Abs [ϵ (M ⁻¹ cm ⁻¹)]	CD [$\Delta\epsilon$ (M ⁻¹ cm ⁻¹)]	MCD [$\Delta\epsilon$ (M ⁻¹ cm ⁻¹)]	$ \Delta\epsilon/\epsilon \times 10^3 $
1	9,600	< 1	< 0.01	-0.94	-
2	14,300	1	0.01	0.55	550
3	15,500	5	0.03	-1.8	360
4	18,200	3	0.06	0.3	100
5	21,200	29	-0.67	0.1	3
6	23,700	67	0.47	0.2	3
7	24,100	< 1	< 0.01	-0.29	-
8	25,500	43	0.1	0.55	13
9	27,600	50	< 0.01	-1.96	40
10	30,000	34	0.01	0.39	11
11	32,500	90	0.1	0.71	8

^aThe full width at half maximum was kept constant at 1,800 cm⁻¹ for bands 1–6 and 8 and 9, at 1,000 cm⁻¹ for band 7, and at 2,100 cm⁻¹ for bands 10 and 11

Table 2

Energies and compositions (%) of the Ni 3*d*- and amide N-based spin-down molecular orbitals (*MO*) based on a spin-unrestricted density functional theory (DFT) computation on the C2S/C6S^{5-c} active-site model

MO	Occupation	E (eV)	Ni ^a	N(His1) ^b	N/O(Cys2) ^c	N(His1) _{eq} ^d	O _z ^e	O _o ^e	Composition
79	0	-4.58	74.6	6.2	6.0	0.0	3.8	0.9	$d(x^2 - y^2)$
78	0	-5.28	73.3	0.7	6.1	5.1	0.1	0.2	$d(z^2)$
77	1	-8.91	9.8	0.2	73.4	0.1	0.5	0.0	N/O(π)
76	1	-9.14	3.4	0.6	78.0	0.0	0.1	0.1	N/O(σ)
74	1	-10.59	53.7	0.1	15.7	2.1	1.0	2.9	$d(xz)$
73	1	-11.01	63.3	3.8	4.4	1.8	11.9	2.6	$d(yz)$
72	1	-11.08	93.0	0.3	1.1	0.4	0.2	0.7	$d(xy)$

MO 75 was not included because it is purely ligand based

^aNi 3*d*, 4*s*, and 4*p* contributions

^b2*s* and 2*p* contributions from the coordinating N atom of the axial imidazole ligand

^c2*s* and 2*p* contributions from the coordinating equatorial N atom of the deprotonated amide group of Cys2

^d2*s* and 2*p* contributions from the coordinating equatorial N atom of the amine group of His1

^e2*s* and 2*p* contributions from the coordinating equatorial O atoms of Ser2 and Ser6

Table 3

Energies and compositions (%) of the Ni 3*d*- and amide N-based spin-down MOs based on a spin-unrestricted DFT computation on the C2S/C6S^{6-c} active-site model

MO	Occupation	E (eV)	Ni ^a	N(His1) ^b	N/O(Cys2) ^c	N(His1) _{eq} ^d	O ₂ ^e	O ₆ ^e	O(H ₂ O) ^f	Composition
84	0	-4.57	76.0	5.5	6.3	0.0	3.4	1.3	0.2	$d(x^2 - y^2)$
83	0	-4.93	78.4	1.0	2.8	5.3	0.2	0.6	1.5	$d(z^2)$
82	1	-9.19	4.0	0.6	76.3	0.0	0.3	0.0	1.9	N/O(π)
81	1	-9.43	6.2	0.5	75.3	0.1	0.1	0.1	0.9	N/O(σ)
79	1	-10.64	53.0	0.2	14.8	5.0	0.6	4.5	0.4	$d(xz)$
78	1	-10.98	72.2	2.6	4.5	1.2	8.2	3.6	0	$d(xy)$
77	1	-11.12	83.9	0.3	3.3	2.1	0.5	0.2	12.3	$d(yz)$

MO 80 was not included because it is purely ligand based

^aNi 3*d*, 4*s*, and 4*p* contributions

^b_{2*s*} and 2*p* contributions from the coordinating N atom of the axial imidazole ligand

^c_{2*s*} and 2*p* contributions from the coordinating equatorial N atom of the deprotonated amide group of Cys2

^d_{2*s*} and 2*p* contributions from the coordinating equatorial N atom of the amine group of His1

^e_{2*s*} and 2*p* contributions from the coordinating equatorial O atoms of Ser2 and Ser6

^f_{2*s*} and 2*p* contributions from the coordinating equatorial O atoms of Ser2 and Ser6 and the axial O atom of water

Table 4

Time-dependent DFT (TD-DFT)-computed ligand-field (LF) transition energies (cm^{-1}) for a series of C2S/C6S^{6-c} active-site models that differ with respect to their Ni–OH₂ bond distances

Band ^a	Ni–OH ₂ bond distance (Å)				Δ^b (cm^{-1})
	2.57	2.72	2.87	3.02	
1	9,900	9,400	9,100	8,800	1,100
2	11,400	11,100	10,800	10,700	700
3	13,400	13,400	13,400	13,400	0
4	16,600	16,000	15,600	15,400	1,200
8	18,000	17,900	17,900	17,800	200
9	21,100	21,100	21,100	21,100	0

^aBands associated with the LF transitions as determined by Gaussian analysis of the C2S/C6S NiSOD Abs, CD, and MCD data

^bDifference between the TD-DFT-computed LF transition energies (cm^{-1}) of the C2S/C6S^{6-c} models in the equilibrium (2.57 Å) and final perturbed (3.02 Å) states