Sulfur K-Edge XAS and DFT Calculations on Nitrile Hydratase: Geometric and Electronic Structure of the Non-heme Iron Active Site

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Abstract: The geometric and electronic structure of the active site of the non-heme iron enzyme nitrile hydratase (NHase) is studied using sulfur K-edge XAS and DFT calculations. Using thiolate (RSO⁻), sulfenate (RSO⁻²), and sulfinate (RSO⁻³)-ligated model complexes to provide benchmark spectral parameters, the results show that the S K-edge XAS is sensitive to the oxidation state of S-containing ligands and that the spectrum of the RSO⁻ species changes upon protonation as the S−O bond is elongated (by ~0.1 Å). These signature features are used to identify the three cysteine residues coordinated to the low-spin Fe³⁺ in the active site of NHase as CysS⁻, CysSO⁻, and CysSO⁻² both in the NO-bound inactive form and in the photolyzed active form. These results are correlated to geometry-optimized DFT calculations. The pre-edge region of the X-ray absorption spectrum is sensitive to the Z_eff of the Fe and reveals that the Fe in [FeNO]³⁺ NHase species has a Z_eff very similar to that of its photolyzed Fe³⁺ counterpart. DFT calculations reveal that this result from the strong π back-bonding into the π* antibonding orbital of NO, which shifts significant charge from the formally t_2g low-spin metal to the coordinated NO.

Introduction

Nitrile hydratase (NHase) hydrolyzes nitriles to amides and is used in the industrial preparation of acrylamide.¹⁻³ The active site can contain either a low-spin Fe³⁺ or a low-spin Co³⁺ ion coordinated by three cysteines and two deprotonated amides (two amides and two cysteines occupying the equatorial plane with an axial cysteine).⁵⁻⁷ The Fe enzyme is isolated in a diamagnetic NO-bound form, activated by irradiation, and is proposed to be stabilized by the presence of butyric acid in the medium.⁴ The sixth coordination site is occupied by a NO (as isolated) or an H₂O molecule (after photolysis).⁸,⁹ In a recent crystal structure of the inactive NO-bound form⁵ (Figure 1a), the cysteine ligands are post-translationally modified to RSO⁻ ligand oxidation states in the active form of the enzyme.¹⁰ Although active sites having transition-metal-

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bound oxidized thiolate residues are rare in nature, mutational studies on these thiolate residues have shown them to be catalytically relevant.¹¹,¹²

The catalytic mechanism of this site is not well understood, and there are several proposals regarding the role of the low-spin Fe⁴⁺ center in activating nitriles for hydrolysis.¹³ The possible functional role of these modified cysteines, and the electronic structure of the as-isolated [FeNO]⁶ complexes (using the Enemark–Feltman nomenclature, where the superscript 6 refers to the total number of valence electrons on the iron and the π* orbitals of NO) and its observed photochemistry, have been a focus of several recent experimental and theoretical studies.¹⁴–¹⁸ A significant number of structurally relevant model complexes have been reported, some of which bind nitriles and some of which reversibly bind NO and show photolytic behavior.¹⁹–²² Furthermore, the study of a series of model complexes having oxidized ligands has shown that this oxidation can significantly alter the pKₐ of a water bound to the low-spin Fe⁴⁺.²³

Ligand K-edge X-ray absorption spectroscopy (XAS) is a direct probe of a ligand’s chemical nature and its bonding to a metal.²⁴,²⁵ The primary transition at the K-edge is 1s → π*. In the case of sulfur, transitions to other unoccupied antibonding orbitals, including the C–S σ*, O–S σ*, and M₄d₃–S antibonding orbitals, gain intensity due to sulfur 3p mixing. The observed intensity of these transitions is then directly proportional to the extent of this mixing.²⁶ This ligand K-edge XAS method has been used to investigate bonding in several models having different types of chlorine- and sulfur-based ligands and in the active sites of NiSOD and nickel dithiolenes, blue copper, Cu₄, and iron–sulfur proteins.²⁷–³² In those studies, where the focus was on the metal–sulfur bonding and its perturbation by the protein environment, it was found that the S K-edge is very sensitive to the Zₚ of a ligand, the chemical environment of the ligand, and the Zₙ of the metal to which it is bound.³³

In this study we apply XAS and density functional theory (DFT) to a series of crystallographically characterized model complexes to identify signature spectroscopic features associated with thiolate-based ligands having different oxidation and protonation states. We then use these features to identify the ligands present in the active site of the protein NHase in its NO-bound inactive and photolyzed active forms. We further investigate the electronic structure of [FeNO]⁶ NHase using DFT calculations in conjunction with the spectroscopic results.

**Experimental Details**

**Sample Preparation.** All four model complexes, Fe⁴⁺(ADIT)₂ (1), Fe⁴⁺(ADIT)(ADIT-O) (2), [Co⁶⁺(η²-SO)(SO₂)₃(Pr,Pr)] (3), and Fe⁴⁺(ADIT-O-ZnCl) (4), were synthesized as reported in the literature.¹⁹,³⁴ For XAS experiments, the samples were grown into a fine powder, dispersed as thinly as possible on sulfur-free Mylar tape in a dry, anaerobic glovebox (N₂) atmosphere, and mounted across the window of an aluminum plate. This procedure has been verified to minimize self-absorption effects. A 6.35-μm polypropylene film window protected the solid samples from exposure to air during transfer from the glovebox to the experimental sample chamber.

The NO-bound NHase protein was expressed and purified as described in ref 35. The protein solutions (in 100 mM phosphate buffer, pH 7.5) were pre-equilibrated in a water-saturated He atmosphere for ~1 h to minimize bubble formation in the sample cell. The solution was loaded via a syringe into a Pt-plated Al block sample holder, sealed in front using a 6.3-μm polypropylene window. To ensure photolysis, the protein sample was illuminated by a 400-W tungsten lamp for 30 min before the XAS data measurements.

**Data Collection and Reduction.** XAS data were measured at the Stanford Synchrotron Radiation Laboratory using the 54-pole wiggler beam line 6-2. Details of the experimental configuration for low-energy studies have been described in an earlier publication.³³ The data collection and error analysis follow the same method discussed previously.³⁶

**Fitting Procedure.** Pre-edge features were fit by pseudo-Voigt line shapes (sums of Lorentzian and Gaussian functions). This line shape is appropriate as the experimental features are expected to be a convolution of a Lorentzian transition envelope and a Gaussian line shape imposed by the spectrometer optics.³⁷,³⁸ A fixed 1:1 ratio of Lorentzian to Gaussian contribution successfully reproduced the pre-edge features. The rising edge was also fit with pseudo-Voigt line shapes. Good fits reproduce the data and its second derivative using a minimum number of peaks. The intensity of a pre-edge feature (peak area) is the sum of the intensity of all the pseudo-Voigt peaks which successfully fit the feature in a given fit. The reported intensity values for the proteins are averages of all good pre-edge fits.

**Computational Details.** All calculations were performed on dual-CPU Pentium Xeon 2.8 GHz workstations using the Amsterdam Density Functional (ADF) program, versions 2004.01, developed by...
Baerends et al.\textsuperscript{39,40} A triple-$\zeta$ Slater-type orbital basis set (ADF basis set TZP) with a single polarization function at the local density approximation of Vosko, Wilk, and Nusair,\textsuperscript{41} with nonlocal gradient corrections of Becke\textsuperscript{42} and Perdew,\textsuperscript{43} was employed. The molecular orbitals were plotted using Molden version 5.1, and the Mulliken\textsuperscript{44} population analyses were performed using the AOMix\textsuperscript{9} program. The calculations of the protein active site were performed using the BP86 functional as well as the hybrid B3LYP\textsuperscript{46} functional with the Gaussian 03 package.\textsuperscript{47} The solvation calculations (single points and optimizations) were performed using the PCM\textsuperscript{48} method and epsilon of 4.0. The optimizations were performed with a mixed basis set with 6-311+g* on Fe, S, N, and O and 6-31g* on C and H. The single-point calculations were performed with a 6-311++g** basis set on all atoms.

**Results and Analysis**

A. S K-Edge XAS of Model Complexes and Free Ligands.

The X-ray absorption spectra of the structurally characterized model complexes $[\text{Fe}^{III}(\text{ADIT})_{2}]^{+}$ (1), $[\text{Fe}^{III}(\text{ADIT})(\text{ADIT-}O)]^{+}$ (2), $[\text{Co}^{II}(\eta^{2}-\text{SO})\text{(SO}_{2})\text{N}_{3}\text{(Pr,Pr)}]^{+}$ (3), and $[\text{Fe}^{III}(\text{ADIT})(\text{ADIT-O-ZnCl}_{3})]^{+}$ (4) (Figure 2) were used as references for ligand oxidation state determination for the NHase enzyme (Figure 1).\textsuperscript{49} Complex 1 contains a low-spin $\text{Fe}^{III}$ atom coordinated by two thiolates, one of which is oxidized in the low-spin complex (2). Complex 4 has a ZnCl$_{3}$ moiety coordinated to the S–O moiety in 2, while 3 has a low-spin $\text{Co}^{III}$ atom ligated by an $\eta^{2}$-coordinated S–O– moiety and an $\text{SO}_{2}$ moiety coordinated through sulfur, as in the proposed active site of NHase (Figure 1).\textsuperscript{3}

The S K-edge XAS of free cysteine at pH 7.0 (black line in Figure 3) has an intense RS$_{-1s}$ transition at 2473.0 eV. The spectra of aqueous solutions of sulfinic acid at pH 12.0 reflect the stabilization of the S1s orbital due to the higher oxidation state of sulfur in the latter.\textsuperscript{3,50} The S K-edge XAS of complex 1 (Figure 3, solid blue) shows two pre-edge transitions around 2470 eV (2469.8 and 2470.5 eV) and a transition at 2473.1 eV, corresponding to RS$_{-1s}$ $\rightarrow$ Fe$_{3d}$ and RS$_{-1s}$ $\rightarrow$ S–C–Co$_{3}$ transitions, respectively. The spectrum of complex 2 (Figure 3, dashed blue) also shows the two pre-edge transitions from the thiolate (now well separated) at 2469.8 and 2470.9 eV and an RS$_{-1s}$ $\rightarrow$ S–C–Co$_{3}$ transition at 2473.3 eV, as well as a new third broad transition with a maximum at 2475.3 eV, which is now assigned to an envelope of the RS$_{-2s}$ transitions. These transitions are shifted higher in energy relative to the RS$_{-1s}$ $\rightarrow$ S–C–Co$_{3}$ transitions due to the deeper energy of the 1s orbital of the oxidized sulfur in RS$_{-1s}$. Note that the RS$_{-1s}$ $\rightarrow$ Fe$_{3d}$ pre-edge transition should also have intensity due to covalent bonding of the $\text{SO}_{2}$ ligand to the metal d-orbitals through the sulfur. However, this would largely overlap the RS$_{-1s}$ $\rightarrow$ S–C–Co$_{3}$ transition at 2473.3 eV in 2. This transition is, in fact, clearly observed at 2473.2 eV for 3 (Figure 3, red), a complex in which there is no RS– ligand and hence no RS$_{-1s}$ $\rightarrow$ S–C–Co$_{3}$ transition. For 2, complex 3 also exhibits RS$_{-2s}$ $\rightarrow$ S–C–Co$_{3}$ and RS$_{-1s}$ $\rightarrow$ S–C–Co$_{3}$ transitions at $\approx$ 2475 eV (maxima at 2474.9 and 2475.9 eV). There is an additional higher-energy feature in the spectrum of 3 at 2478.8 eV, which is assigned to the RS$_{-2s}$ $\rightarrow$ S–C–Co$_{3}$ and RS$_{-1s}$ $\rightarrow$ S–C–Co$_{3}$ transitions. Note that this transition envelope is shifted up in energy by almost 2 eV for complex 3 (2478.8 eV, Figure 3, red) relative to free sulfinic acid (2470.0 eV, Figure 3, solid gray). This shift has contributions from both the increase in $Z_{\text{eff}}$ of sulfur in $\text{SO}_{2}^{\ddagger}$ due to its covalent bonding to the $\text{Co}^{III}$ ion, which shifts the sulfur 1s orbital to deeper energy, and from the shorter S–O bonds in 3 (1.45 Å) as compared to the 1 in the free ligand (1.51 Å), which shifts the $\sigma^{*}$ orbital to higher energy (Table 1). The XAS of complex 4 (Figure 3, green) is very similar to that of 2 (Figure 3, dashed blue) and has the same assignment of features, i.e., RS$_{-1s}$ $\rightarrow$ Fe$_{3d}$ and RS$_{-1s}$ $\rightarrow$ S–C–Co$_{3}$ transitions.
The calculated $S_{3p}$ characters were scaled up by an approximate factor of $K$ (K-edge). Though the absolute transition energies were underestimated (due to lack of relativistic corrections), the relative energies of the pre-edge and the rising edge features are in good agreement with the experimental spectra in Figure 3.

The intensity distribution for 2 (Figure 4, dashed blue) shows two features corresponding to $RSO_2^{-1s} \rightarrow Fe^{3d}$ and $RSO_2^{-1s} \rightarrow RS^{-1s} \rightarrow RS^{-C=S-}$ transitions at 4.0 eV (first transition split due to ligand field effects on the 3d orbitals) and 7.0 eV, respectively. It also shows a third feature at 9.0 eV which corresponds to contributions of the $RSO_2^{-1s} \rightarrow RSO^{-C=S-}$ and $RSO_2^{-1s} \rightarrow RSO^{-O=S-}$ transitions. Calculations for complex 3 give the $RSO_2^{-1s} \rightarrow CO_3^{2-}$ transition at 6.3 eV and the $RSO_2^{-1s} \rightarrow RSO^{-C=S-}$ and $RSO_2^{-1s} \rightarrow RSO^{-O=S-}$ transitions at 9.0 eV (Figure 4, red). Note that the $\sigma^*$ transitions are separated in energy by 1 eV and that the calculations reproduce the experimentally observed energy splitting of this feature. There is an additional higher-energy feature for 3 (Figure 4, red), corresponding to the overlapping contributions from $RSO_2^{-1s} \rightarrow RSO^{-C=S-}$ and $RSO_2^{-1s} \rightarrow RSO_2^{-O=S-}$ transitions. Calculations on both free $CH_3SO_2^{-}$ and $CH_3SOH$ show this feature at 9±11 eV (Figure 4, solid gray and dashed gray, respectively). These calculations reproduce the 2-eV increase in energy of this feature observed in the data for 3 relative to that of the free ligand and show no significant effect of protonation of the oxygen of the $RSO_2^{-}$ group on the $S_{3p}$ distribution, consistent with experiment (Figure 3, solid gray and dashed gray). A calculation performed on the $CH_3SO_2^{-}$ fragment, having the geometry of complex 3 (i.e., with shorter $S-O$ bond lengths) but not coordinated to a metal ion, shows no significant shift of the $RSO_2^{-1s} \rightarrow RSO_2^{-C=S-}$ and $RSO_2^{-1s} \rightarrow RSO_2^{-O=S-}$ transition energies, indicating that the experimentally observed 2-eV shift of this feature between the free ligand and complex 3 (Figure 3, red and gray lines) is a result of $Co^{II}$ coordination to the sulfur of $RSO_2^{-}$. This shifts charge density from $S$ to $Co^{II}$, stabilizing the $S_{1s}$ orbital energy in complex 3 relative to that of the free ligand.

A DFT calculation on $CH_3SO^{2-}$ shows one feature at 7.0 eV that corresponds to an envelope of the $RSO_2^{-1s} \rightarrow RSO^{-C=S-}$ and $RSO_2^{-1s} \rightarrow RSO^{-O=S-}$ transitions (Figure 4, green). In contrast to calculations on $RSO_2(H)$, the DFT calculation on the free $CH_3SO$ ligand shows dramatic differences in the $S_{3p}$ energy/intensity distribution relative to that of the $CH_3SO_2^{-}$ ligand. The $RSO_2^{-1s} \rightarrow RSO^{-C=S-}$ and $RSO_2^{-1s} \rightarrow RSO^{-O=S-}$ transitions, which combine into one peak at 7.0 eV in the deprotonated ligand (Figure 4, solid green), are now split into two peaks (Figure 4, dashed green) at 6.0 eV (the $RSO_2^{-1s} \rightarrow RSO^{-O=S-}$ transition) and 8.0 eV (the $RSO_2^{-1s} \rightarrow RSO^{-C=S-}$ transition) upon protonation. Geometry-optimized DFT calculations on these free ligands (Table 1) show that the $C-S$ bond

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Table 1. DFT-Optimized Geometric Parameters

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<td>$M-S$</td>
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<td>1.84 (1.82)</td>
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<tr>
<td>$C-S$</td>
<td>2.19 (2.17)</td>
<td>1.85 (1.84)</td>
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<td>1.87 (1.87)</td>
</tr>
<tr>
<td>$C-S$</td>
<td>2.20 (2.16)</td>
<td>1.84 (1.84)</td>
</tr>
<tr>
<td>$S-O$</td>
<td>1.86</td>
<td>1.60</td>
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(51) The calculated $S_{3p}$ characters were scaled up by an approximate factor of 2 for every two units of change in its oxidation number to account for the increase in $Z_{int}$, which will increase the transition dipole integral, i.e., $(S_{1s}f S_{3p})$, leading to an increase in absorption intensity.

(52) This transition is not shifted in energy in the $CH_3SO_2^{-}$ fragment, having the geometric parameters of complex 2, but is shifted by 1.6 eV to 8.9 eV in 2, reflecting the shift of charge from the coordinated sulfur atom due to covalent interaction, as also observed in complex 3.
shortens by 0.06 Å and the S–O bond elongates by 0.1 Å on protonation of CH$_3$SO$^–$. This shifts the C–S σ* orbital higher and the O–S σ* orbital lower in energy, resulting in the observed energy splitting of these features. A similar elongation of the S–O and shortening of the C–S bonds is observed in complexes 4 and 3 (O–S 1.56 Å, C–S 1.86 Å and O–S 1.57 Å, C–S 1.87 Å, respectively) relative to 2 (O–S 1.55 Å, C–S 1.85 Å). Thus, the experimentally observed energy splitting of the 2474–2476 eV features of complexes 3 and 4 is due to the weakening of the S–O and strengthening of the C–S bonds as a result of the metal coordination to the RSO$^–$ oxygen atom. The larger energy splitting for complex 4 is probably due to its stronger coordination to Zn$^{II}$. As shown in Figure 4, full protonation produces the largest effect. It should be noted that for RSO$_2$(H) the changes in bond lengths with protonation are within 0.01 Å (Table 1). In summary, from the model studies it is found that the S1s → C–S σ* and S–O σ* transitions are very sensitive to the Z$_{eff}$ of the sulfur. RS$^–$ has RS$^{–1s}$ → Fe$_{3d}$ and RS$^{–1s}$ → RS$^{–C–O}$ transitions at ~2470 and ~2473 eV, respectively, RSO$_2$ has RSO$^{–1s}$ → RSO$^{–C–O}$ and RSO$^{–1s}$ → RSO$^{–O–S\delta^–}$ transitions at 2475 eV which split by 2 eV on protonation, and RSO$_2^–$ has the RSO$_{2}^{–1s}$ → RSO$_{2}^{–C–S\delta^–}$ and RSO$_{2}^{–1s}$ → RSO$_{2}^{–O–S\delta^–}$ transitions at 2477–2479 eV (depending on metal ion coordination) which are not significantly affected by protonation.

C. S K-Edge XAS of Nitrile Hydratase. The S K-edge XAS of the inactive NO-bound form of NHase shows four distinct features (Figure 5, black). The lowest-energy feature, at 2470.1 eV (inset), is assigned as the RS$^{–1s}$ → Fe$_{3d}$ transition. The broad envelope at 2472–2474 eV can be assigned as a composite of RS$^{1s}$ → RS$^{–C–S\delta^–}$ transitions of the ligated cysteine, two free cysteines and nine methionines. The other higher-energy features, at 2476.1 and 2478.5 eV, are relatively weak; however, a simulated spectrum generated by the normalized addition of the spectra of aqueous methionine and cysteine at pH 7.5 and 1, representing thiolate coordination but no oxidized sulfur (Figure 5, blue), shows no higher-energy features. Alternatively, simulation including the oxidized ligands (Figure 5, red, complex 3) shows two higher-energy features at 2475–2476 and 2475.5–2479.5 eV, similar to those for NHase-NO at 2476.1 and 2478.5 eV, indicating that they are associated with the oxidized sulfur ligands present in the NO-bound form of the protein. The feature at 2478.5 eV for the enzyme can be assigned to RSO$_2^{–}$. Note that this is at a higher energy than for the RSO$^{–1s}$ → RSO$_2^{–C–S\delta^–}$ and RSO$_2^{–1s}$ → RSO$_2^{–O–S\delta^–}$ transitions observed for the free RSO$_2^–$ ligand (at 2476–2478 eV, Figure 3, gray) but lower than those for the Co$^{III}$ complex 3 (at 2478.8 eV, Figure 3, red), indicating coordination of the CysSO$_2^{–}$ ligand at the protein active site but with a weaker bond relative to complex 3.

The feature at 2476.1 eV could originate from RSO$^{–}$, although this feature is shifted to higher energy by ~1 eV than is observed for RSO$^{–}$ in complexes 2 and 3. In sections A and B (vide supra), it is shown that the energy of the RSO$^{–}$ feature is sensitive to the chemical environment of the oxygen. Inspection of the second derivatives of the absorption data (where minima in second derivative represent maxima in the absorption spectrum) (Figure 6) reveals that NHase-NO enzyme data has a minimum at 2476.1 eV (Figure 6, black), while the RSO$^{–}$-ligated complex 2 has a broad minimum at 2475.2 eV (Figure 6, dashed blue). In 4 (Figure 6, green), where Zn$^{II}$ is strongly bound to the O of RSO$^{–}$, the RSO$^{–1s}$ → RSO$^{–C–S\delta^–}$ feature has shifted down to 2474.8 eV, reflecting a weaker S–O bond in 4, while the RSO$^{–1s}$ → RSO$^{–C–S\delta^–}$ negative feature remains at 2476.1 eV. As found in section B, protonation of RSO$^{–}$ will shift the S–O σ* transition to 2 eV lower in energy than for the Co$^{III}$ complex 3, due to the elongation of the S–O bond. Hence, the absence of a single minimum in the second derivative, corresponding to a peak in absorption between 2474 and 2475 eV in the NHase data, argues against the presence of an RSO$^{–}$ residue and indicates that the cysteine sulfenic acid residue in the NHase active site is protonated. While the RSO(H)$_{1s}$ → RSO(H)$_{C–S\delta^–}$ transition is observed in the data for NHase (Figure 6, black) at 2476.1 eV, about 1.0 eV higher than the single peak observed for 2 (which can be used as an estimate of the energy position of a pure RSO$^{–}$ feature), the RSO(H)$_{1s}$ → RSO(H)$_{S–O\delta^–}$ transition of NHase, which is estimated to be 2 eV lower in energy relative to the RSO(H)$_{1s}$ → RSO(H)$_{C–S\delta^–}$ transition, could be merged into the RS$^{–1s}$ → RS$^{–C–S\delta^–}$ transition envelope at 2472–2474 eV.

Note that there is a minimum in the protein XAS data at 2474 eV, which may originate from RSO$^{–1s}$ → RSO$^{–S–O\delta^–}$. However, in the presence of nine methionine residues this feature is not observed in the absorption data.
S K-edge XAS data for the photoactivated protein (Figure 5, dashed black) show no significant change relative to the data for the inactive NO-bound NHase. This indicates that the oxidation states of the metal and the sulfur ligands and the protonation state of $\text{RSO}_2^-$ are virtually identical in the NO-bound inactive and the photolyzed active enzyme. However, there is a markedly increased pre-edge intensity upon photolysis (Figure 5, inset), which is analyzed in section E.

D. DFT Calculations on the Active Site of NHase. While the S K-edge XAS data for the protein active site and model complexes enabled us to assign the chemical nature of two of the three cysteinyl ligands ($\text{CysS}^-$ and $\text{CysSOH}$), it could not help to discern the protonation state of the $\text{CysSO}_2^-$ ligand. Geometry-optimized DFT calculations were performed on the crystallographically characterized NO-bound inactive form to evaluate this issue (Figure 7A). The active site was modeled using two oxidized cysteines coordinated to the Fe in the equatorial plane (Cys 114, Cys 112) and a methyl thiolate modeling the axial cysteine (Cys 109). There are two hydrogen bonds from nearby arginine residues (Arg 141 and Arg 56) which are modeled by two $\text{C(NH}_3^+ \text{)}$ fragments. The optimized structures show that the $\text{CysSO}_2^-$ remains in its ionized form.

(55) The central C of the $\text{C(NH}_3^+ \text{)}$ species was frozen during optimization.
This is reasonable as the pKₐ of RSO₂⁻ is 6−7 and that of arginine is 12−14; hence, they are expected to be ionized under the experimental conditions. Also, in a vacuum the charged species should neutralize while they may not do so in a PCM calculation. On the contrary, the proton from the C(NH₂)₃⁺ fragment, H-bonding to CysSO⁻, shifts to the CysSO⁻ oxygen, producing a neutral CysSOH species (Figure 7B). This computational model agrees well with the geometric structure obtained from the XAS data as the optimized geometry displays a long (1.68 Å) S−O bond upon protonation, which will split the RSO⁻ → RSO⁻−C−S⁻ and RSO⁻ → RSO⁻C−S⁺, as observed experimentally. However, it must be noted that the pKₐ of the RSO⁻ residue is 10−11 and that for arginine is 12−14.56 As indicated above, the protonation state of the arginine-CysSO unit will depend on the dielectric constant of the medium. Geometry optimizations were also performed using a PCM solvation model with an epsilon of 4.0. The results of this optimization show that the CysSO⁻ and the C(NH₂)₃⁺ residues remain charge-separated after optimization (Figure 7C), in contrast to the vacuum-based calculations and the experimental results from XAS. However, the energy difference between the CysSOH−C(NH₂)₃⁺(NH) and the CysSO−C(NH₂)₃⁺ forms is only 4 kcal/mol and depends on modeling of the active site and the functional used (4 kcal/mol for B3LYP and 7 kcal/mol for BP86).57 This energy difference corresponds to a pKₐ difference of 3 units. An electrostatic calculation (not included in the DFT modeling) of the pKₐ’s for the buried arginines in NHase shows that these vary over the range 16.6 (the surface residues have ~12) to 8.5, with one of the two (Arg 167) H-bonded to the SO⁻ ligand having a pKₐ of 9.3.58

### E. Pre-edge Analysis

Due to covalent interaction with the axial CysS⁻, the unoccupied 3d orbitals of the NO-bound NHase active site will have Sₚ mixing, and hence will exhibit RS₁ → Fe₃d transition intensity, which is proportional to the coefficient of Sₚ in these orbitals. The S K-edge XAS of the NO-bound inactive form has a distinct pre-edge feature with a maximum at 2470.0 eV (Figure 6, inset, black). The renormalized intensity of this pre-edge feature is 0.69 unit, which correspond to 20% Sₚ character mixed into the antibonding 3d orbital (Table 2). The NHase-photoactivated sample has two pre-edge features, one at 2469.6 eV and a second at 2470.2 eV, with 0.31 and 0.51 unit of intensity, respectively (Figure 6, inset, dashed black). This indicates that the active site of the photoactivated form has two 3d antibonding orbitals with 9% and 15% Sₚ character (Table 2).

### Table 2. Pre-edge Analysis Results for NHase Data

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<th>t₂&lt;sub&gt;eff&lt;/sub&gt;</th>
<th>e&lt;sub&gt;eff&lt;/sub&gt;</th>
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<td>NHase-NO</td>
<td>2470.1</td>
<td>20</td>
</tr>
<tr>
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<td>2470.2</td>
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</table>

The MO diagrams of the NO-bound ([FeNO]) and photolyzed NHase are given in Figure 8 (the β unoccupied orbitals). Figure 8 (right) shows that the NO-bound form has a formal t₂<sup>4</sup> configuration. There are two unoccupied NO π<sup>u</sup> LUMOs and two higher-energy unoccupied e orbitals, d<sub>f</sub> and d<sub>xy</sub>. The d<sub>f</sub> orbital (highlighted in blue) has 19% Sₚ character due to pseudo-σ (Sₚ orbital slightly tilted off the Fe−S bond as the angle C−S−Fe > 90°) overlap with the thiolate, which would provide the intensity for a single pre-edge feature, as observed in Figure 6 (inset) at 2470.1 eV. Photolysis cleaves the Fe−NO bond and creates a low-spin Fe<sup>II</sup> center with a H₂O/OH⁻ molecule coordinated in place of NO. Here the t₂ hole is oriented by the strong axial CysS⁻ donor into the d−π orbitals (shaded in red). This covalent mixing of the CysS⁻ π into the d orbital leads to the new lower-energy feature at 2469.5 eV in the pre-edge of the photolyzed protein. The higher-energy e feature remains at 2470.2 eV, as the pseudo-σ overlap with this axial CysS⁻ ligand does not change.

The pre-edge region of the S K-edge XAS data is affected by the chemical nature of the ligand, the ligand field of the metal, and its Z<sub>eff</sub>. Here we use the pre-edge to probe the Z<sub>eff</sub> of the metal ion and thus obtain insight into its effective oxidation state. It is important to note that Fe<sup>II</sup> complexes do not show a pre-edge transition, as the Z<sub>eff</sub> for the Fe<sup>II</sup> is low and this shifts the Fe₃d manifold of orbitals to higher energy, such that the RS₁ → Fe₃d transitions overlap with the RS₁ → RS<sub>C−S⁻</sub> transition and are not distinguishable. The fit to the NHase data shows a small pre-edge feature at 2470.1 eV (Figure 5 inset, Table 2), which suggests that the Z<sub>eff</sub> of the Fe in the [FeNO]<sup>+</sup> active site of NHase is significantly higher than that for low-spin Fe<sup>II</sup> complexes and similar to that of Fe<sup>III</sup> NHase.

### Discussion

S K-edge XAS is very sensitive to the chemical nature of the sulfur atom. In this study we have used structurally characterized synthetic model complexes to identify signature transitions corresponding to different types of sulfur ligands. Not only does the edge show dramatic differences upon oxidation; in the case of RSO⁻ the edge is also sensitive to protonation. The data for both the NO-bound inactive and photolyzed active forms of the NHase enzyme show the presence of three types of sulfur ligands at the active site: CysS⁻, CysSOH, and CysSO₂H. Although we could not identify the protonation state of the CysSO₂⁻ using our data, DFT calculations in both the gas phase and PCM show that it is ionized (Figure 9). This model can now be used to understand the role of these oxidized cysteines in catalysis.

The oxidation of the cysteine ligands has been proposed to be catalytically relevant, either by affecting the binding of a nitrite, followed by a nucleophilic attack by H₂O, or by affecting the binding of a H₂O to the open coordination site of the Fe, which then ionizes to release a H⁺ that catalyzes the hydrolysis. A geometry-optimized DFT calculation on a low-spin Fe<sup>III</sup> NHase active site with three thiolates (Figure 10) and using H₂O as the sixth ligand shows weak binding (Fe−OH₂ = 3.4

(57) Given the resolution of the crystal structure, it is not possible to distinguish between calculated structures B (e = 0) and C (e = 4.0) using structural parameters.
(58) Estimated using the crystal structure of NO bound Nhae (pdb id 2AHJ) with the software PRGPKA: Li, H.; Robertson, A. D.; Jensen, Jan H. Proteins 2005, 61, 704−721.
(59) The absence of a pre-edge feature in Fe<sup>III</sup> sites has been studied in complexes with weak field ligands (Cl⁻, R⁻). Here, the 3d manifold in the NHase-NO complex will be further shifted to higher energy by the strong amide and NO donors. Hence, the pre-edge transition will shift deeper into the RS₁ → RS<sub>C−S⁻</sub> transitions of the rising edge. Thus, the spin state cannot account for the low pre-edge energy.
Å) affinity. However, calculations with the oxidized ligand set show favorable H₂O binding in this vacant coordination site (Fe–OH₂ = 2.1 Å). Thus, the oxidized sulfur ligands are weaker donors, which will increase the Lewis acidity of the Fe³⁺ center and thus tune the ligand binding affinity to this vacant, catalytically relevant, exchangeable coordination site.

The electronic structure of linear [FeNO]⁶ units has been a major focus of research in bio-inorganic chemistry. Münck and co-workers, using Mössbauer spectroscopy, suggested that the electronic structure of the NO-bound NHase can be described as Fe(IV)-NO⁻. That conclusion was based on small isomer shifts (0.03–0.05 mm/s) and high quadrupole splittings (1.3–2.0 mm/s) observed for an [FeNO]⁶ model. However, the electronic properties of these complexes, investigated by recent DFT calculations, have suggested that this unit can be described as a low-spin Fe²⁺ coordinated to a NO⁺ unit. On photolysis, a low-spin Fe³⁺ center is produced which is well-characterized by EPR, Mössbauer, and resonance Raman spectroscopy. Note that, from the XAS near-edge transitions, we know that the chemical nature of the CysS⁻ does not change upon photolysis, and hence it serves as a spectator of the change in Zeff of the Fe ion in this process. The pre-edge of this photolyzed ferric active site...
site is observed at 2469–2470 eV (Figure 6, inset, dashed black), an energy very similar to that of the pre-edge of the inactive NO-bound form of NHase. This suggests that the \( Z_{\text{eff}} \) of the Fe atom in the [FeNO]\(^6\) NHase active site is very similar to that of Fe\(^{III}\) NHase rather than to that of Fe\(^{II}\) or Fe\(^{IV}\). Also note that the second derivative of the NO-bound form (Figure 6 inset, black) has only one feature at 2470.1 eV, corresponding to one RS\(^-\)\(1s \rightarrow Fe_{3d}(e)\) transition, while the second derivative of the photolyzed protein has an additional feature in the pre-edge at 2469.5 eV, corresponding to the creation of a new low-energy t\(_2\) hole in forming the low-spin Fe\(^{III}\) center. This suggests that the [FeNO]\(^6\) unit of inactive NHase has a \( Z_{\text{eff}} \) similar to that of Fe\(^{III}\) but does not have a t\(_2\) hole. Such an electronic structure would be consistent with strong back-donation from the low-spin t\(_2\) iron to NO \(\pi^*\) orbitals. From Figure 8, the inactive NO-bound form has a low-spin t\(_2\) iron center that has 28–31% back-bonding interaction with the NO (3d character summed over two unoccupied NO \(\pi^*\) orbitals), which shifts charge density from the formally Fe\(^{II}\) and increases its \( Z_{\text{eff}} \).

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**Supporting Information Available:** Complete ref 47; optimized coordinates of complexes 1–4, free ligands, and the NO active site of NHase (in gas phase and using the PCM model). This material is available free of charge via the Internet at http://pubs.acs.org.

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