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The Electronic Structure of the Metal Active Site Determines the Geometric Structure and Function of the Metalloregulator NikR

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Abstract

NikR is a nickel-responsive metalloregulator protein that controls the level of Ni^{2+} ions in living cells. Previous studies have shown that NikR can bind a series of first row transition metal ions, but only binds to DNA with high affinity as a Ni^{2+} complex. To understand this metal selectivity, S K-edge XAS of NikR bound to different metal ions was used to evaluate the different electronic structures. The experimental results are coupled with DFT calculations on relevant models. This study shows that both the Z_{eff} of the metal ion and the donor nature of the ligands determine the electronic structure of the metal site. This impacts the geometric structure of the metal site and thus the conformation of the protein. This electronic structure contribution to geometric structure can be extended to other metal selective metalloregulators.

Graphical Abstract

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Supporting Information

The supporting information is available free of charge on the ACS publication website.

Geometry optimized Ni(II) site for the native Ni_NikR protein (Figure S1); 2nd derivatives of the XAS spectra (Figure S2); Geometry optimized Co(II) and Cu(I) variant for the NikR site (Figure S3 and S4); and the Cartesian coordinates for the computational models.

Introduction

Ni is essential for many microorganisms as a cofactor for several key enzymes,^{1, 2} but high cellular concentrations of Ni²⁺ ions are toxic.³ NikR is a nickel-responsive transcriptional regulator that represses the expression of an importer of Ni²⁺ ions in bacterial cells, NikABCDE, in response to Ni binding.^{4–10} When the availability of Ni²⁺ ions in the cell is too high, Ni²⁺ ions load the NikR protein, which enhances its binding to a palindromic operator site on the upstream section of DNA encoding the NikABCDE operon. This inhibits RNA polymerase binding and thus turns off the expression of the Ni importer in order to maintain Ni²⁺ homeostasis.

Experiments have shown that a series of 3d transition metals can bind to the NikR protein, and that their binding affinity follows the Irving-Williams series: Mn²⁺ < Co²⁺ < Ni²⁺ < Cu²⁺ > Zn²⁺.^{7, 11–13} Of these metals, only Ni²⁺ binding gives rise to repression of the Nik importer.⁴ Metal specificity arises from a number of factors including the relative affinities of all the metal sensors in the cell, which keep certain metals from being detected by a specific metallosensor, the cytoplasmic metal-buffer capacity, and the allosteric effects induced by binding the cognate metal ion(s).^{14–17} These allosteric changes drive an increase or decrease in DNA binding affinity,^{18, 19} depending on the function of the transcriptional regulator. The structure of NikR has been determined by x-ray crystallography in apo-, metal-bound forms, and with DNA bound.^{20–24} The protein belongs to the ribbon-helix-helix structural family of DNA-binding proteins and binds Ni²⁺ in a planar four-coordinate site at a subunit interface. (The ligands are His87, His89, and Cys95 from one subunit and His 76 from another; *E. coli* numbering). The Drennan group has suggested that the ordering of the α 3 helices, which is dependent on using the Ni²⁺ to form a crosslink between subunits, plays a key role in NikR-DNA binding.⁶ (Figure 1)

There are many experimental results that support this idea. Currently available crystal structures include apo-NikR (*e.g.*, PDB: 1Q5V, 2BJ3), Ni(II)-NikR (*e.g.*, PDB: 2HZA, 2BJ9)⁶, Cu(II)-NikR C-terminal domain²⁵ (PDB: 3BKT) and Zn(II)-NikR C-terminal domain²⁵ (PDB: 3BKF). Both the Ni(II)-NikR and Cu(II)-NikR C-terminal domains have a planar four-coordinate metal site with 1Cys and 3His ligands from two different subunits. The Zn-NikR crystal structure showed two possible Zn binding sites near the thiolate ligand. Both sites have low coordination number and neither forms the crosslink between subunits. The crystal structures showed that only the Ni-NikR and Cu(II)-NikR have ordered α 3 helices.²⁵ This is further supported by CD spectra which show that Ni(II)-NikR and Cu(II)-NikR have similar amounts of α helix, while the apo-NikR and Zn-NikR have less. EXAFS analysis also showed that non-cognate metals bind to NikR using different coordination numbers and geometries, and showed that Cu(I)-NikR has a coordination number of three, likely similar to the Zn²⁺ site, and that the Co²⁺ NikR complex has a higher coordination number of six.⁴ Hydrogen-deuterium exchange experiments showed that Ni-NikR and Cu(II)-NikR have similar conformations, while the rates of H/D exchange are quite different for Co-NikR, Zn-NikR and apo-NikR.⁴

The prior results summarized above are from spectroscopic studies, biochemical assays, and crystal structures. Here, we evaluate the metal ion specificity based on the electronic

structures of the transition metal-ligand bonds in NikR in order to understand the role of electronic factors in the metal-specific regulation mechanism that may be extended to other metal ion regulator proteins.

S K-edge X-ray Absorption Spectroscopy (XAS) has been developed by our group to experimentally determine the electronic structures of open shell metal-sulfur bonds.^{26–28} High energy X-ray radiation from a synchrotron excites electrons from the core levels (S 1s) into the valence level (Ψ^*). When the S containing ligand forms a covalent bond with a transition metal, the antibonding valence orbitals are a linear combination of the metal 3d and S 3p orbitals, $\Psi^* = \sqrt{1 - \alpha^2} M_{3d} - \alpha S_{3p}$. The energy of this transition reflects the energy difference between the S 1s and Ψ^* , which is mainly shifted by the metal 3d energy, reflecting the effective nuclear charge (Z_{eff}) of the transition metal ion. Because the S 1s orbital is localized on the sulfur, and the S 1s $\rightarrow$ S 3p transition is electric dipole allowed, the intensity of the pre-edge transition reflects the covalency (α^2) of this bond.²⁹ Thus S K-edge XAS is a powerful technique to directly probe bond covalency, and can be used to study electronic structure contributions to metal ion binding and activation.²⁶

The present study applies S K-edge XAS to probe the Co, Ni, Cu and Zn NikR M-S(Cys) bonds. This is compared to calculated electronic structures to explore the covalent contributions to metal site geometry, and in particular why Ni^{2+} , but not other transition metal ions, generates a biological response.

For the transition metal ions included in this study, Cu^+ and Zn^{2+} have d^{10} configurations, thus both have no pre-edge features. Cu^{2+} has a d^9 configuration and a doublet ground state. Ni^{2+} has a d^8 configuration, thus the ground state could be a singlet or a triplet, denoted as low spin and high spin, respectively. Similarly, Co^{2+} has a d^7 configuration, and the ground state could be either doublet (low spin) or quartet (high spin). In this study, all possible spin states are evaluated computationally.

Materials and Methods

Sample preparation. *E. coli* NikR was overexpressed in *E. coli* DL41(DE3) plysS cells and was isolated as previously described.¹ Apo-NikR was prepared by incubating the protein with 50 mM EDTA at 4°C for 5 days. The protein was dialyzed against 20 mM Tris (pH 8.3) and 600 mM NaCl (Buffer A). 0.8 – 1.0 mole-equivalents of an aqueous solution of 10 mM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, or $\text{CuCl}_2 \cdot 6\text{H}_2\text{O}$ (Fisher Scientific) solution were added to apo-NikR and the samples were incubated overnight at 4°C. NiNikR was prepared in buffer A with 1mM EGTA and was desalted twice into buffer A. The protein metal content was determined using a Perkin-Elmer Optima DV4300 ICP-OES instrument. Protein concentrations were determined in 6 M guanidine hydrochloride using the extinction coefficient $\epsilon_{276} = 4490 \text{ M}^{-1}\text{cm}^{-1}$. The metal:protein ratios for samples prepared in buffer A are as follows: 0.55:1 Co, 0.71:1Ni, 0.49:1 Cu, and 0.59:1 Zn. Samples of the metallated protein were concentrated to 4 – 8 mM and then used for sulfur K-edge spectroscopy.

S-K-edge spectroscopy

Sulfur K-edge XAS spectra were measured at the Stanford Synchrotron Radiation Lightsource (SSRL) beam line 4–3. Details of the beam line configuration was described in our earlier papers.^{27, 28} The protein solutions were loaded into 50 μ L teflon cells, with a thin polypropylene film as the front window, and S-free Kapton tape as the back window. A shutter was installed to minimize possible photoreduction when the monochromator changes its position. The photon energy was calibrated using the first pre-edge peak of standard Na₂S₂O₃•5H₂O as 2472.02 eV.²⁶ Three scans were measured on each sample and for each species a different prep were measured at two different beamtimes to ensure reproducibility. Raw XAS data were calibrated and averaged using MAVE in the EXAFSPAK package.³⁰ The background were removed from all spectra by the PySpline software.³¹ The spectrum was normalized to 1.0 at 2490.0 eV. The pre-edge features were fitted with peaks having pseudo-Voigt shapes with a fixed Lorentzian to Gaussian ratio of 1:1, using the EDG_FIT program in the EXAFSPAK package.³⁰ The areas under the pre-edge were converted to %S 3p character based on our previous paper.²⁹ The error from the data processing is ~5%. Only the sulfur that binds to the transition metal contributes to the pre-edge intensity. To account for the metal loading, the processed data were renormalized by $[Intensity - (1 - x) * Apo] / x$, where x is the metal loading ratio ($0 < x < 1$), Intensity is the XAS intensity at each energy, and Apo is the XAS intensity of the Apo protein at the corresponding energy. The renormalized data are plotted together with the pre-edge intensity expanded in Figure 2. Because NikR has 1 additional Cys and 2 Met that are not bound to the metal, the final covalency values are multiplied by 4.

Computational Models

DFT calculations with spin polarization were performed using Gaussian 09 package³², with the hybrid functional B3LYP, and 6–311G(d) basis sets on all atoms. The coordinates of metal ion and α -Carbons of the ligands were set to freeze according to the crystal structure (PDB code 2HZV) during geometry optimizations. In the model using F⁻ ion to replace the Cys ligand, the F-M-O bond angles were fixed to avoid the formation of strong O-H...F hydrogen bonds. The %S was obtained by the CSPA population analysis, using QMForge software.^{33, 34}

Results

NikR S K-edge XAS

The S K-edge XAS spectra of a series of NikR samples, including the apo protein and proteins loaded with Co(II), Ni(II), Cu(II) and Zn(II) are shown in Figure 2. The metal loading ratio for Co-NikR is 50%, for Ni-NikR is 72%, for Cu-NikR is 41% and for Zn-NikR is 54%, as determined by ICP-OES (*vide infra*). The loading ratio has been accounted for in the data processing; the low loading ratios for Co-NikR and Cu-NikR results in the lower S/N of their spectra. Both Ni-NikR and Cu-NikR have well defined pre-edge features, which correspond to S 1s $\rightarrow$ metal 3d transitions. Apo- and Zn-NikR have no pre-edge features, due to the lack of the 3d electron holes. It should be noted that the Co-NikR spectrum has a weak but reproducible feature at ~ 2471.2eV, which is further supported by

the second derivative of this spectrum (SI). This feature indicates that a Co-S(Cys) bond exists in this protein complex.

The pre-edge integrated intensities can be converted into M-S bond covalencies²⁹ *i.e.*, the amount of S character mixed into the frontier “d” molecular orbitals. These are given in Table 1, %S column, Exp rows. The Co-S covalency is 5% S 3p character summed over unoccupied d orbitals, which is considerably less than that for the Ni-S covalency (22%) and the Cu-S covalency, which is the highest (33%). Their pre-edge energies, which represent the energy gap between S 1s and metal 3d levels, decrease in the order: 2171.2 eV for Co-NikR, 2170.5 eV for Ni-NikR, and 2469.8 eV for Cu-NikR. Both the trends in intensities and the peak energies reflect the change in the effective nuclear charge (Z_{eff}) of the metals, $Z_{\text{eff}}(\text{Co}) < Z_{\text{eff}}(\text{Ni}) < Z_{\text{eff}}(\text{Cu})$. These trends are qualitatively consistent with our previous experimental spectra on blue copper related models.¹³

Analysis

In order to further understand the spectroscopic features and the electronic structures of the active sites, DFT calculations were performed on the Co^{2+} , Ni^{2+} and Cu^{2+} NikR models. Truncated simplified structures were based on that of Ni-NikR with one CH_3S^- and three imidazole ligands bound in a planar structure.⁶ All geometry optimizations were performed allowing for the possible coordination of two additional water ligands at axial positions. This reflects previous EXAFS results that showed a coordination number of six for the Co^{2+} complex of *E. coli* NikR.⁴ The two axial water ligands model possible protein derived ligands including Y60, E97, or other carboxyl or amide groups in the vicinity of the metal site. The geometry optimizations were performed with Cartesian coordinates of the metal and α carbons of the four ligands fixed, to model the protein constraints. Frequency calculations and population analyses were performed on the optimized structures. For the Ni^{2+} and Co^{2+} complexes, both high-spin and low-spin states were evaluated.

For Cu(II)-NikR, a four-coordinate planar active site was obtained, which is consistent with the crystal structure of the C-terminal metal binding domain of NikR.²⁵ The metal-ligand bond distances are also consistent with experiment (Table 1).

For Ni(II)-NikR, the low-spin Ni ($S=0$) site optimized to a four-coordinate planar geometry, while the high-spin Ni ($S=1$) site optimized to a six-coordinate octahedral geometry. The high-spin site has longer metal-ligand bonds in the equatorial plane, as expected for the $(dz^2)^1(d_{x^2-y^2})^1$ configuration. Based on the coordination number, bond distances and calculated Ni-S bond covalency, the low-spin Ni(II)-NikR geometric and electronic structures are more consistent with the experimental results (Table 1). The low-spin state is also calculated to be 3.3 kcal/mol lower in free energy relative to the high-spin state. Also the crystal structure of the native Ni(II) bound NikR is square planar⁶ and all such Ni(II) structures have $S=0$ ground state.

For Co(II)-NikR, the low-spin Co ($S=1/2$) site optimized to a four-coordinate planar geometry, while the high-spin Co ($S=3/2$) site favors a six-coordinate octahedral geometry. Again, the high-spin site has longer metal-ligand bonds in the equatorial plane. Based on the

coordination number, bond distances and Co-S bond covalency values, the high-spin six-coordinate Co(II)-NikR is more consistent with experimental results (Table 1). The high-spin state is also calculated to be 1.8 kcal/mol lower in total energy relative to the low-spin state. Also, the EPR spectrum of Co(II)-NikR collected at 12 K shows features that are typical of an $S = 3/2$ Co(II) center³⁵.

From the above results, the Ni(II)-NikR site favors a low-spin four-coordinate structure, while the Co(II)-NikR site favors a high-spin six-coordinate geometry. These results are supported by the S K-edge XAS data. This difference could derive from the difference in electron-electron repulsion between d^7 and d^8 configurations, or from the difference in covalency (the Ni-S bond is much more covalent than Co-S bond, 22% vs 5%, respectively, Table 1). To uncouple the relative contributions of these factors, we constructed a 4His + 2H₂O model by replacing the Cys ligand with a His and performed parallel calculations.

In the Co[His₄(H₂O)₂]²⁺ model, the high-spin six-coordinate site is also favored, by 1.3 kcal/mol. Comparing this result to that obtained for His₃Cys ligation, the thiolate ligand does not play a significant role in determining the ground state. Although the Co-S bond is more covalent in the low-spin calculation (13% vs 5% S p character), the two additional axial ligands in the high-spin complex provide comparable stabilization.

In the Ni[His₄(H₂O)₂]²⁺ model, the high-spin six-coordinate site is favored by 3.1 kcal/mol. From correlation to the Ni[His₃Cys] model where the low-spin four-coordinate site is favored by 3.3 kcal/mol, the thiolate ligand stabilizes the low-spin state. This derives from the strong CysS⁻ σ donor interaction with the Ni²⁺. The MO contour of the $d_{x^2-y^2}$ unoccupied orbital is shown in Figure 3. This structure is stabilized by σ donation from the thiolate with 22% S 3p character from S K-edge XAS. The calculated value is 18%.

To further evaluate whether the effect of the thiolate is from the covalency of the M-S(Cys) bond or the negative charge of this ligand, we performed another set of parallel calculations using a M[His₃(H₂O)₂F]⁺ (M = Ni²⁺, Co²⁺) model. In order to avoid formation of strong O-H...F hydrogen bonds, F-M-O bond angles were fixed during the optimization. The results parallel those of the M[His₄(H₂O)₂]²⁺ calculations but with the high-spin six-coordinate structures even more favored (7 kcal/mol for Co²⁺, 10 kcal/mol for Ni²⁺, vs 1.3 and 3.3 kcal/mol, respectively). Thus, the higher covalency of the Ni-S(Cys) bond due to the higher Z_{eff} of Ni(II) leads to the selectivity associated with the low-spin four-coordinate structure.

Discussion

Metal selectivity in the allosteric response of metallosensors has been attributed to coordination number/geometry^{19, 36–38} and to ligand selection by different metals.^{4, 39} This study shows that within the set of ligands available for coordinating metals in a given protein, the resulting differences in electronic structures are also important factors in determining the coordination number and geometry and thus play a role in discriminating between metals. Thus, nature may employ different ligand sets to confer preferences for different metal geometries, and thus different specificities, via electronic structure contributions to bonding. In the case of NikR, the presence of a single Cys thiolate ligands

favors a low-spin four-coordinate geometry for Ni^{2+} that is not favored in the Co^{2+} complex, which instead adopts a six-coordinate structure.⁴ Both metals coordinate the single conserved CysS-donor in the metal binding site. This was shown by EXAFS analysis for the Ni^{2+} complex, but was not detected by EXAFS for the Co^{2+} complex.⁴ However, the results of the analysis of the S K-edge XANES reported here show a pre-edge feature for Co-NikR (Figure 2) that indicates that Co^{2+} does indeed bind to a Cys similar to the other metal ions. The Co-S interaction was probably not detected in the Co K-edge EXAFS results³⁵ because of the long Co-S bond calculated for the high-spin six-coordinate structure (Table 1) that could lead to a high σ^2 value in the EXAFS data fitting.

Cu^+ and Zn^{2+} are d^{10} and thus do not have a pre-edge in the S K-edge XAS. From the crystal structure by the Drennan group²⁵, Zn^{2+} has 2 possible binding sites, both with low coordination numbers. For Cu^+ , there is no crystal structure available, but EXAFS results published by the Maroney group suggest a 3 coordinate Cu^+ site⁴. From the DFT calculations starting from an initial 6 coordinate Cu^+ site, then geometry optimized to a 3 coordinate final structure (Figure S4), consistent with the EXAFS results.

The discrimination between Co^{2+} and Ni^{2+} in the monothiolate coordination environment of NikR is due to the higher covalency of the Ni-S(Cys) bond with respect to the Co-S(Cys) bond, which drives from the higher Z_{eff} of Ni^{2+} . Thus, the interaction of the metal center with the single thiolate ligand is critical in determining the coordination number/geometry of the NikR complex. The lower coordination number and planar geometry of the Ni^{2+} complex are apparently important to the metal-specific biological response in NikR. It is proposed that the crosslink of the two units through the metal ion can stabilize the $\alpha 3$ helices in a protein conformation that enables DNA binding.⁶ In Co-NikR, the Co-S bond is weak, which leads to a high-spin six-coordinate metal site structure.⁴ This is quite different from the highly covalent Ni-S bond that leads to the four-coordinate planar metal site found in the native Ni-NikR.⁴ This structural difference may perturb the local H-bonds and thus impact the tertiary structure of the protein.

Interestingly, another metallosensor with monothiolate coordination of metals, RcnR, exists.^{35, 39} *E. coli* RcnR is also a transcriptional regulator involved in maintaining Ni^{2+} homeostasis, but de-represses transcription of an exporter (RcnAB) in response to cognate metal binding.³⁵ In this case, both Co^{2+} and Ni^{2+} are bound in six-coordinate mono-thiolate sites,³⁵ and the sensor responds similarly to the binding of either metal ion.³⁵

The 6 coordinated Ni(II)RcnR site is high spin from the magnetic susceptibility data⁴⁰ and has a long Ni-S bond (2.6 Å) according to the EXAFS.³⁵ This is not consistent with the results of the present study of Ni(II)-NikR (low spin, 4 coordinate, 2.2 Å Ni-S bond) and suggests that the CysS-Ni bonding in RcnR is limited by protein constraints. A possible contribution to this is the orientation of the $\alpha\text{C-S-Ni}$ angle relative to the equatorial ligand plane, as this dihedral angle has been found to impact the d orbital splittings which can affect the spin state of Ni(II).⁴¹ However, with no crystal structure of RcnR available, this possibly cannot be further evaluated. Nevertheless, the E63C mutation that changes one of glutamate to a thiolate shifts the Ni site to low spin with a short Ni-S bond.⁴⁰ The presence

of the in plane strong Ni-S bond favors the low spin Ni site with lower coordination number as found here from Ni(II)-NikR.

Alternatively, WT Co(II)-RcnR is also 6 coordinated and high spin,³⁵ which is consistent with our results on Co(II)-NikR. It does have a shorter Co-S distance relative to Ni(II)-RcnR, suggesting a stronger bond and less protein constraint. This is consistent with the difference observed in ligation of Ni(II) vs Co(II) by RcnR. The situation with respect to Co²⁺ sensing by the Ni²⁺ metallosensors, NikR and RcnR, is ideal for *E. coli*, which does not synthesize vitamin B₁₂, and therefore has no reason to import Co²⁺ and every reason to export this unneeded transition metal ion. Also, other noncognate metals such as Cu(I) and Zn(II) bind to RcnR with lower coordination number and do not elicit a biological response.³⁹

The trend in increasing Z_{eff} continues for Cu(II), and gives rise to a highly covalent Cu-S bond (Table 1), and experiments show that both Cu(II)-NikR and Ni(II)-NikR complexes have similar DNA binding affinities (or the order of 10^{-9} M), both over 10 fold stronger than with other metal complexes.⁴² This likely accounts for the observed planar Cu[His₃Cys]⁺ complex in NikR and for the planar Cu(His₂Cys₂) site characterized for InrS.⁴⁰ InrS is a Ni²⁺-responsive member of the RcnR-CsoR family of metalloregulators that is found in cyanobacteria and de-represses expression of the Ni²⁺ exporter, NrsD, in response to binding Ni²⁺.⁴³ Similar to NikR, but in contrast to the monothiolate complex in the orthologous RcnR, Ni²⁺ also adopts a planar Ni(His₂Cys₂) structure in InrS, and the metallosensor response *in vitro* is not metal-specific. Selectivity between Ni²⁺ and Cu²⁺ is not important in a biological context because copper is trafficked as Cu⁺ ions in the reducing environment of the cell, and Cu availability is controlled and kept under the sensitivity (binding affinity) of the Ni trafficking system by the Cu metallosensors, which have higher affinity for Cu⁺ ions than do the proteins involved in cellular Ni²⁺ trafficking.¹⁷

In summary, the highly covalent Ni-S bond leads to the 4-coordinate square planar metal site in the native Ni-NikR. However when the metal ion is changed to Co²⁺, the weaker Co-S bond results in a high spin 6-coordinate metal site structure. The coordination number decreases to 3 or less for Zn²⁺ and Cu⁺ because of their mostly ionic nature. These structural differences likely perturb the local H-bonds and thus impact the tertiary structure of the NikR protein and its interaction with DNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expansion of the NikR-DNA binding region based on PDB code 2HZV. The DNA, two $\alpha 3$ helices, and one Ni site with 4 ligands from 2 different subunits are shown. Another Ni site is faded in the back.

Figure 2,
S K-edge XAS of the metal varied NikR proteins, with the pre-edge region enlarged in insert.

Figure 3.
LUMO of the low-spin Ni-NikR active site, mainly showing the $d_{x^2-y^2} \sigma^*$ interaction with the ligands.

Table 1

Experimental and DFT Results of Different Metal Site Models

M		M-S (Å) ^b	M-N (Å) ^b	M-OH ₂ (Å)	Coord #	%S	Relative energy (kcal/mol) ^c
Ni	Exp	2.13/2.22	2.01/1.85		4	22 ± 3	
	DFT s ^a	2.23	2.1/1.9	>3	4	18	0
	DFT t	2.44	2.2/2.0	2.24	6	12	3.3
	4His s		1.92	>3	4		0
	4His t		2.12	2.18	6		-3.1
	3His 1F-s		2.0/1.9	2.80/3.20	4		0
	3His 1Ft		2.3/2.0	2.14	6		10
Co	Exp	-	2.04/2.21	2.17	6	5 ± 1	
	DFT d	2.26	2.04/1.98	>3	4	13	0
	DFT q	2.35	2.21/2.11	2.2/2.3	6	4	-1.8
	4His d		1.99	2.45	4		0
	4His q		2.16	2.23	6		-1.3
	3His 1F-d		2.2/1.9	2.3	4		0
	3His 1F-q		2.3/2.1	2.2	6		7
Cu	Exp	2.21/2.43	2.4/2.0		4	33 ± 5	
	DFT d	2.35	2.25/2.0	>3	4	26	

^a Spin states used in the calculation. s for singlet; d for doublet; t for triplet; and q for quartet.^b Experimental bond distances are from published EXAFS or crystallography results.^c A value of 0 indicates that this is the energy reference state.