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Glutamate Ligation in the Ni(II) and Co(II) Responsive *Escherichia coli* Transcriptional Regulator, RcnR

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Abstract

Escherichia coli RcnR (resistance to cobalt and nickel regulator, *EcRcnR*), is a metal-responsive repressor of the genes encoding the Ni(II) and Co(II) exporter proteins RcnAB by binding to P_{rcnAB} . DNA-binding affinity is weakened when the cognate ions Ni(II) or Co(II) bind to *EcRcnR* in a six-coordinate site that features a (N/O)₅S ligand donor-atom set in distinct sites: while both metal ions are bound by the N-terminus, Cys35, and His64, Co(II) is additionally bound by His3. On the other hand, the non-cognate Zn(II) and Cu(I) ions feature a lower coordination number, have a solvent-accessible binding site, and coordinate protein ligands that do not include the N-terminal amine. A molecular model of apo-*EcRcnR* suggested potential roles for Glu34 and Glu63 in binding Ni(II) and Co(II) to *EcRcnR*. The roles of Glu34 and Glu63 in metal binding, metal selectivity and function were therefore investigated using a structure/function approach. X-ray absorption spectroscopy (XAS) was used to assess the structural changes in the Ni(II), Co(II), and Zn(II) binding sites of Glu → Ala and Glu → Cys variants at both positions. The effect of these structural alterations on the regulation of P_{rcnA} by *EcRcnR* in response to metal binding was explored using LacZ reporter assays. These combined studies indicate that while Glu63 is a ligand for both metal ions, Glu34 is a ligand for Co(II) but possibly not for Ni(II). The Glu34 variants affect the structure of the cognate metal sites, but they have no effect on the transcriptional response. In contrast, the Glu63 variants affect both the structure and transcriptional response, although they do not completely abolish the function of *EcRcnR*. The structure of the Zn(II) site is not significantly perturbed by any of the Glu variations. The spectroscopic and functional data obtained on the mutants were used to calculate models of the metal site structures of *EcRcnR*.

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Supplementary materials and methods, tables, and discussion of modeling results, XAS of E34A- and E34C-RcnR bound to Co(II) in Cl buffer, pre-edge features of Ni(II) complexes, Evans magnetic susceptibility measurements, lacZ titrations, and SEC, fluorescence anisotropy data, and tables with additional fits for WT and mutant *EcRcnR* proteins. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

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bound to Ni(II), Co(II) and Zn(II). The results are interpreted in terms of a switch mechanism, in which a subset of the metal binding ligands is responsible for the allosteric response required for DNA release.

TOC Figure

Transition metals are essential for the function of a wide variety of proteins and are thus critical for the maintenance of proper cellular metabolism.^{1–3} However, excessive metal levels are toxic.^{4–8} It is therefore imperative that cells tightly regulate their intracellular metal content, which is often achieved by employing a cellular trafficking system composed of metal-binding proteins. The complexity of these systems is dependent on the requirements of the cell and on the metal ion in question. Typically, transition metal trafficking systems are equipped with importers, exporters, metallochaperones and accessory proteins required for metal insertion and metallocenter assembly, as well as regulatory proteins that control the cellular levels of the other trafficking proteins.⁹ A common arrangement in bacteria employs metal-responsive transcriptional regulators to control the expression of metal importers and exporters.^{2, 4, 10} These transcriptional regulators are metal-specific, responding only to a single “cognate” metal or a limited number of such metals. How these proteins distinguish cognate metal(s) from non-cognate metals with similar physical properties is not completely understood.^{2, 9–15}

E. coli has no confirmed cobalt-containing proteins,¹⁶ and while it can utilize vitamin-B₁₂ if it is available, it cannot synthesize it *de novo*.^{17–19} In contrast, *E. coli* requires nickel for the active sites of at least three anaerobically expressed hydrogenases, and thus has a dedicated nickel trafficking system for maintaining nickel homeostasis.^{20–23} This trafficking system consists of an importer, NikABCDE,^{24, 25} chaperones (*e.g.* HypA for hydrogenase-3),^{26–28} accessory proteins involved in hydrogenase metallocenter assembly (HypB and SlyD),^{29, 30} and an exporter, RcnAB.^{31, 32} In addition, there are two homotetrameric transcriptional regulators: *EcNikR*,^{33, 34} which controls the expression of the Ni(II) importer NikABCDE by increasing the affinity for DNA in the Ni(II)-bound form, and *EcRcnR*,^{35, 36} which controls the expression of the Ni(II) and Co(II) exporter RcnAB by binding to DNA in the apo-form and decreasing the affinity for DNA when bound to either Ni(II) or Co(II). RcnAB and RcnR are so far the only known proteins involved in Co(II) trafficking in *E. coli*.

RcnR is a founding member of a family of entirely α -helical transcriptional regulators.³⁷ This family is now known to be widespread across prokaryotes and senses a wide variety of substrates, including Cu(I) (CsoR and RicR),^{38, 39} Ni(II) (RcnR and InrS),^{35, 40} sulfide (CstR),⁴¹ and formaldehyde (FrmR).^{42, 43} *EcRcnR* recognizes a TACT-G₆-N-AGTA DNA

sequence, of which there are two in the *rcnA-rcnR* intergenic region.⁴⁴ In addition to its recognition sequence, tetrameric *EcRcnR* interacts with the flanking DNA regions, leading to DNA wrapping.⁴⁴

EcRcnR binds a variety of metal ions *in vitro*, but only Ni(II) and Co(II) binding results in transcription of *rcnAB in vivo*.⁴⁴ Previous studies have established that cognate and non-cognate metals are distinguished in part by coordination number, a common feature of specific metal recognition in other metal-responsive transcriptional regulators,^{10, 45, 46} as well as by ligand selection.^{47, 48} Cognate metals were shown to bind to *EcRcnR* in six-coordinate sites composed of (N/O)₅S ligand donor-atoms, while non-cognate metals consistently showed lower coordination numbers.³⁵ Ligand selection also plays a role in the *EcRcnR* response. *EcRcnR* binds both cognate and non-cognate metals using the only cysteine in the protein, Cys35,³⁵ but cognate metals are distinguished from non-cognate metals by inclusion of the N-terminal amine in the coordination complex, thus accounting for the difference in coordination number of cognate versus non-cognate metals.⁴⁷ Consistent with this model, when the Co(II)-binding His3 residue was mutated to a Glu residue, Zn(II) can occupy a six-coordinate binding site and the protein is now responsive to Zn(II) in the cell.⁴⁷

The Co(II)- and Ni(II)-bound forms of *EcRcnR* are distinguished in at least two ways: His3 is a ligand for Co(II) and not for Ni(II), and, in addition, the M-S(Cys35) distance differs greatly depending on the metal ion (Ni(II) = 2.6 Å and Co(II) = 2.3 Å).^{47, 48} It has been previously suggested that metal/small molecule binding residues can be identified in members of the CsoR/RcnR family of transcriptional regulators by utilizing a W-X-Y-Z fingerprint motif.^{37, 49} In *EcRcnR*, these positions are filled by His3, Cys35, His60, and His64, although His3 is not a Ni(II) ligand. However, there appears to be diversity in metal site structures and functions across the currently known members of the CsoR/RcnR family of transcriptional regulators. Two recently reported phylogenetic analyses and sequence alignments of the CsoR/RcnR family members reveal no clear distinction between the amino acids in the canonical W-X-Y-Z fingerprint that denotes first coordination sphere residues of this family for Ni(II) and Cu(I) sensors.^{49, 50} For example, the Ni(II) sensor RcnR contains some of the same fingerprint motifs as the Cu(I) binding proteins CsoR and RicR, yet has a different metal binding motif than InrS, another known Ni(II)-responsive protein. Thus, as previously observed,⁵⁰ fingerprint analysis of this family of regulators is a poor way to identify the cognate metals. Metal site structures also do not help to identify the cognate metals, as *SyInrS* and *EcRcnR* both respond to Ni(II) binding but adopt distinct four-coordinate planar and octahedral geometries, respectively.^{35, 40} Thus, each protein employs different structure/function relationships, although there is an emerging trend in terms of protein function in that regulators of importers (*e.g.*, InrS, NikR) employ four-coordinate Ni(II) sites, while regulators of exporters, such as RcnR, employ six-coordinate Ni(II) sites.

No structure of *EcRcnR* is yet available, but the protein shows homology to CsoR, a tetrameric copper sensing transcriptional regulator of the same family.⁵¹ Recent structural bioinformatics studies have provided a computational model of apo-*EcRcnR* in the tetrameric state,⁵² in agreement with experimental data.³⁵ The structural model is consistent with the presence of three α -helices in each monomer (Figure 1A). The three α -helices form

large contact surfaces that contribute to the building up of the dimer and the tetrameric oligomer is obtained by dimerization of the two dimers through the N-terminal helices. Figure 1B shows that the model of apo-*EcRcnR* features a large number of putative metal-binding residues localized at the interface between two monomers in the vicinity of Cys35 from one chain: the N-terminal amine, the imidazole rings of His3, His64 and His67, as well as His60, known to be involved in metal binding, are all found in this region. In addition, Glu34 and Glu63 are also located in this potential metal loading region (Figure 1B), and their carboxylate groups could act as ligands for Co(II) and Ni(II). Although not strictly conserved in the CsoR/RcnR family of transcriptional regulators, these glutamate residues lie in X-space and Z-space in the W-X-Y-Z spatial motif.

In order to test the involvement of these two residues in metal-binding and effects on DNA-affinity, X-ray absorption spectroscopy (XAS) was used to assess the metal site structure in Co(II), Ni(II), and Zn(II) complexes of the *EcRcnR* variants E34A-RcnR and E63A-RcnR (loss of ligand variations) as well as E34C-RcnR and E63C-RcnR (ligand substitution variations). The results of XAS analysis confirm that both of these carboxylates are ligands in the Co(II) complex, while for Ni(II), only Glu63 unambiguously acts as a ligand. Neither Glu residue is found to be involved in Zn(II) binding. Promoter-lacZ fusion transcription reporter assays show that Glu34 is not critical for *EcRcnR* function, while Glu63 RcnR variants show a decreased response to both Ni(II)- and Co(II)-binding. The structures of *EcRcnR* bound to Ni(II), Co(II) and Zn(II) were modeled according to the spectroscopic and functional experimental data, providing a working hypothesis for understanding the geometrical arrangement of the metal-binding ligands, a type of information not available from EXAFS analysis alone. The structures identify other putative ligands, and further understanding of the relationships between the metalation state and the DNA-binding capability of *EcRcnR*.

MATERIALS AND METHODS

EcRcnR mutagenesis

Point mutations for producing E34A-, E34C-, E63A-, and E63C-*EcRcnR* protein variants were introduced into the wild-type *EcRcnR* gene on a pET22-b vector (pRcnR³⁶) using the QuikChange (Stratagene) site-directed mutagenesis method. The same method and primers were used in order to introduce the point mutations on to the pJ114^{35, 36, 44} vector for LacZ transcriptional assays. The mutations for E34A-RcnR and E34C-RcnR were made using PCR and employing the primers atgctcgacgagccgcacgcacatgcgctgcagttttac and atgctcgacgagccgcactgttgcgctgcagttttac and their reverse complements, respectively. The mutation for the E63A-RcnR variant was made using the primers cggaagtgattaaaggtcatctgacggcacacatc and catccccctggtgaacgatgtgtgcgcgcag, while the mutation for the E63C-RcnR variant was made using the primers cggaagtgattaaaggtcatctgacgtgccacatc and catccccctggtgaacgatgtgtgcgcgcag. The presence of the desired mutation was confirmed in each case via sequencing at GENEWIZ Inc. (South Plainfield, NJ).

RcnR expression and purification

Expression of all *EcRcnR* variants was performed as previously described.^{47, 48}

Chromatographic purification of *EcRcnR* employed an AKTA-FPLC system (Amersham Biosciences). Lysate from 1 L of cells was loaded onto a 5 mL HiTrap Heparin HP column (GE Life Sciences) equilibrated with 20 mM HEPES, 1 mM TCEP, 5 mM EDTA, 10% glycerol, 300 mM NaCl at pH 7.0 (buffer A). The protein was washed with buffer A and then eluted in one step using 30% buffer B (20 mM HEPES, 1 mM TCEP, 5 mM EDTA, 10% glycerol, 1 M NaCl at pH 7.0). The protein was eluted in four 5 mL fractions and the fraction containing *EcRcnR*, as determined by SDS-PAGE, was then split into two 2.5 mL fractions and loaded on a 120 mL HiLoad 16/60 Superdex 75 (GE Life Sciences) column equilibrated with buffer A. Fractions were collected on the basis of absorbance at 280 nm. An SDS-PAGE gel was used to determine which fractions contained *EcRcnR*. The fractions containing *EcRcnR* were exchanged into buffer B containing 200 mM NaCl and loaded onto a 5 mL SP Sepharose HP column. The column was washed with buffer C (20 mM HEPES, 1 mM TCEP, 5 mM EDTA, 10% glycerol, 50 mM NaCl at pH 7.0) and then the protein was eluted in a single step using 40% buffer B and 60% buffer C in three fractions. The purity of the fractions was checked using SDS-PAGE. Fractions containing *EcRcnR* were estimated to be >90% pure and were used without further purification. The expected molecular weights of the purified proteins were confirmed using electrospray ionization mass spectrometry (ESI-MS) using an ABI-SCIEX QSTAR-XL equipped with a turbo-spray ESI source in sequence with an Agilent 1100 HPLC system with a BioBasic-8 (Thermo Scientific) column for salt removal. Alternatively, samples were measured by direct injection following exchange into 200 mM ammonium acetate using Zeba desalting column. The calculated/observed monomer molecular weights of wild-type (10002.7/10002.6 Da), E34A- (9944.7/9945.0 Da), E34C- (9976.7/9975.9 Da), E63A- (9944.7/9945.1 Da), and E63C- (9976.7/9976.8 Da) *EcRcnR* proteins confirm that the desired proteins were obtained.

Preparation of metal complexes

All proteins were concentrated to ~150 μ M except where noted, and the solution was exchanged twice using a buffer containing 20 mM HEPES, 300 mM NaBr, 1 mM TCEP, 10% glycerol at pH 7.0 (buffer M). The exchange was performed using 10 mL Zeba spin desalting columns (Thermo Scientific) with a 7 kDa molecular weight cutoff to remove EDTA and exchange NaCl for NaBr. The two samples in NaCl (E63A-RcnR with Co(II) and Ni(II)) were prepared using the same procedure except for the use of 300 mM NaCl instead of NaBr in the buffer. NaBr was used in XAS experiments to distinguish cysteine S-donors from Cl⁻ ligands in the EXAFS analysis, and to identify solvent accessible sites when Br⁻ binding to the metal occurs. Protein concentrations were confirmed using the experimentally determined extinction coefficient $\epsilon_{276} = 2530 \text{ M}^{-1} \text{ cm}^{-1}$ from protein denatured with 8 M guanidine hydrochloride.³⁵ Four aliquots of a 50 mM stock solution in water providing a total of 2.5 equivalents of Ni(OAc)₂ or Co(OAc)₂ (Fisher Scientific) or 1.5 equivalents of Zn(OAc)₂ (J. T. Baker) were added to the apo-*EcRcnR* solutions to produce the respective metal complexes. The concentrations of the metal ion stock solutions were determined by using a Perkin-Elmer Optima DV4300 ICP-OES instrument. The protein samples were allowed to equilibrate overnight with the added metal ions before being incubated with

Chelex (Sigma Aldrich) beads for ~30 minutes to remove nonspecifically bound metal ions. The protein solutions were then analyzed for metal content by ICP-OES. This analysis was performed by preparing a 1 ppm sample of the *EcRcnR* protein complexes using the previously determined protein concentrations. The metal:protein (monomer) ratios for samples prepared in buffer M were as follows: WT-*EcRcnR*, 1.2:1 for Ni(II), 0.8:1 for Co(II), 0.7:1 for Zn(II); E34A-*EcRcnR*, 0.4:1 for Ni(II), 0.6:1 for Co(II), 0.5:1 for Zn(II); E34C-*EcRcnR*, 0.2:1 for Ni(II), 1:1 for Co(II), 0.7:1 for Zn(II); E63A-*EcRcnR*, 0.4:1 for Ni(II), 0.3:1 for Co(II), 0.3:1 for Zn(II); E63C-*EcRcnR*, 0.5:1 for Ni(II), 0.2:1 for Co(II), 0.6:1 for Zn(II).

X-ray Absorption Spectroscopy (XAS)

Samples of the metallated proteins were concentrated to ~1–4 mM and a total volume of ~50 μ L in buffer M (unless otherwise noted) using microspin concentrators (Millipore). The concentrated samples were injected into polycarbonate XAS sample holders wrapped in kapton tape, and then rapidly frozen in liquid nitrogen and stored at -80°C .

XAS data for the Co(II) E63C-*EcRcnR* complex and Ni(II) complexes of WT-, E34A-, E34C-, E63A-, E63C-*EcRcnR*, and Zn(II) complexes of E34C-, E63A-, and E63C-*EcRcnR* were collected on beam line X3B at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratories (Upton, NY). Data for the Co(II) complexes of WT-, E34A- E34C- E63A-, and E63A- (in NaCl buffer), for the Ni(II) complex of E63A- in NaCl buffer, and for the Zn(II) complex of WT- and E34A-*EcRcnR* complexes were collected at the Stanford Synchrotron Radiation Laboratory (SSRL) on beam line 9–3. For data collected at NSLS, the samples were cooled to ~50 K using a He dispex cryostat, and data was collected under ring conditions of 2.8 GeV and 120–300 mA using a sagittally focusing Si(111) double-crystal monochromator. X-ray fluorescence was collected using a 30-element Ge detector (Canberra). Scattering was minimized by placing a Z-1 filter between the sample chamber and the detector. For data collected at SSRL, the samples were cooled to ~10 K using a liquid helium cryostat (Oxford Instruments). The ring conditions were 3 GeV and 450–500 mA. Beam line optics included a Si(220) double-crystal monochromator. X-ray fluorescence data was collected using a 100-element detector (Canberra) at beam line 9–3 or a 30 element detector (Canberra) at beam line 7–3. Soller slits with a Z-1 element filter were placed between the sample chamber and the detector to minimize scattering.

Internal energy calibration was performed by collecting spectra simultaneously in transmission mode on the corresponding metal foil to determine the first inflection point on the edge, which was set to 7709.5 eV for Co(II), 8331.6 eV for Ni(II), or 9660.7 eV for Zn(II). X-ray absorption near-edge structure (XANES) data were collected from -200 to $+200$ eV relative to the metal K-edge. Extended X-ray absorption fine structure (EXAFS) data were collected to $15k$ above the reference edge energy.

Data Reduction and Analysis

The SixPack software⁵³ program was used to remove bad channels, average the data, and to perform energy calibrations, in addition to data reduction and normalization. Edge normalization and background subtraction was performed using a Gaussian pre-edge

function and a seven spline point quadratic polynomial between $k = 2 \text{ \AA}^{-1}$ and $k = 14 \text{ \AA}^{-1}$ for the post-edge region, followed by normalization of the edge jump.

The Artemis software program was used for EXAFS analysis. Artemis employs the IFFEFIT engine to fit EXAFS data using parameters generated by FEFF6.^{54, 55} The data were

converted to k -space using the relationship: $k = [\frac{2m_e(E - E_0)}{\hbar^2}]^{1/2}$. The k^3 -weighted data were Fourier-transformed over a k -range of 2–12.5 $\text{\AA}^{-1}$ using a Kaiser-Bessel window for all data sets, and fit in r -space using an S_0 value of 0.9. The r -space spectra shown in the figures are not phase-corrected.

The Artemis software program was used for EXAFS analysis by utilizing the EXAFS equation with parameters generated using FEFF6.^{54, 55} The EXAFS equation is defined as:

$$\chi(k) = \sum_i \frac{N_i f_i(k) e^{-2k^2 \sigma_i^2}}{k r_i^2} \sin[2k r_i + \delta_i(k)]$$

where $f(k)$ is the scattering amplitude, $\delta(k)$ is the phase-shift, N is the number of neighboring atoms, r is the distance to the neighboring atoms, and σ^2 is the disorder to the nearest neighbor. Data sets were fit with separate sets of r_{eff} and σ^2 for the sulfur, nitrogen, and imidazole rings with initial values of 0.0 $\text{\AA}$ and 0.003 $\text{\AA}^2$, respectively. Each fit was initiated with a universal E_0 (7723 eV for Co, 8340 eV for Ni, and 9670 eV for Zn) and an initial E_0 of 0 eV, the latter of which was allowed to vary for each fit. In order to assess multiple-scattering contributions from histidine imidazole ligands, fits were performed over an r -space range of 1–4 $\text{\AA}$. Histidine ligands were fit as geometrically rigid imidazole rings with varied angles of rotation, α , with α being defined as the rotation around an axis perpendicular to the plane of the ring and going through the coordinated nitrogen. In this manner, the distances of the five non-hydrogen atoms in the imidazole ring were fit in terms of a single metal-N bond distance for various angles ($\alpha = 0 - 10^\circ$).^{56–58} Multiple-scattering parameters for imidazole ligands bound to nickel, cobalt and zinc were generated using the FEFF6 software package with the initial input obtained from average bond lengths and angles gathered from crystallographic data, as previously described.^{56, 59} All paths with relative amplitudes larger than 16% of the pathway with maximum intensity were included.

To assess the goodness of fit from different fitting models, the fit parameters χ^2 , reduced χ^2 ($r\chi^2$), and r -factor were minimized. Increasing the number of adjustable parameters is generally expected to improve the R -factor; however χ^2 may go through a minimum, the increase indicating that the model is overfitting the data. These parameters are defined as follows:

$$\chi^2 = \frac{N_{\text{idp}}}{N_{\varepsilon^2}} \sum_{i=1}^N (\{\text{Re}[\tilde{\chi}_{\text{data}}(R_i) - \tilde{\chi}_{\text{theory}}(R_i)]\}^2 + \{\text{Im}[\tilde{\chi}_{\text{data}}(R_i) - \tilde{\chi}_{\text{theory}}(R_i)]\}^2)$$

and

$$r\chi^2 = \frac{\chi^2}{N_{\text{idp}} - N_{\text{var}}}$$

where N_{idp} is the number of independent data points defined as $N_{\text{idp}} = \frac{(2\Delta r \Delta k)}{\pi}$, Δr is the fitting range in r -space, k is the fitting range in k -space, $N_{\text{e}2}$ is the number of uncertainties to minimize, $\text{Re}()$ is the real part of the EXAFS Fourier-transformed data and theory functions, $\text{Im}()$ is the imaginary part of the EXAFS Fourier-transformed data and theory functions, and $\tilde{\chi}(R_i)$ is the Fourier-transformed data or theoretical function.

Additionally, IFEFFIT calculates the R -factor for each fit, which is directly proportional to χ^2 and is the root-mean-square difference between the data and calculated fit, and given by:

$$R = \frac{\sum_{i=1}^n \{[\text{Re}[\tilde{\chi}_{\text{data}}(R_i) - \tilde{\chi}_{\text{theory}}(R_i)]]^2 + [\text{Im}(\tilde{\chi}_{\text{data}}(R_i) - \tilde{\chi}_{\text{theory}}(R_i))]^2\}}{\sum_{i=1}^n \{[\text{Re}(\tilde{\chi}_{\text{data}}(R_i))]^2 + [\text{Im}(\tilde{\chi}_{\text{data}}(R_i))]^2\}}$$

Magnetic susceptibility measurements

Evans' NMR method⁶⁰ was used to measure the magnetic susceptibility of Ni(II) complexes (2.25 mM Ni(II) WT-*EcRcnR* and 0.939 mM E63C-*EcRcnR*) that were buffer exchanged into 20 mM HEPES, 150 mM NaCl, 1 mM TCEP, 5% glycerol, pH 7.0 in 90% H₂O/10% D₂O. The protein solutions (400 μ L) were placed into an NMR tube and a coaxial insert containing the 10% deuterated buffer was placed inside the NMR tube. Relative shifts between the DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) reference peak at 0 ppm and the peak shifted due to the magnetic properties of the protein sample in the outer NMR tube were used to calculate μ_{eff} using equations:^{61, 62}

$$\chi_g = \frac{-3\Delta\nu}{4\pi\nu_o m}$$

$$\chi_M = \chi_g M$$

$$\chi'_M = \chi_M - \sum \chi_L$$

$$\mu_{\text{eff}} = 2.83 \sqrt{\chi'_M T}$$

where χ_g is the gram susceptibility in cgs units, χ_M is the molar susceptibility, χ'_M is the molar susceptibility corrected for the diamagnetic contribution of the protein, μ_{eff} is the magnetic moment in Bohr Magnetons, m is the concentration of the sample in g/mL, ν is

the peak separation in Hz, ν_o is the frequency of the spectrometer in Hz (400.132471×10^6 Hz), M is the molecular weight of the protein in g/mol, χ_L is the diamagnetic correction for the protein, and T is the temperature (298 K). The diamagnetic correction was determined by measuring ν for apo-protein at the same concentrations and using that to calculate the molar susceptibility of apo-protein.

LacZ reporter assays

LacZ reporter assays were carried out as previously described⁶³ with the following modifications. Starter cultures of *E. coli* strain PC113 (*rcnR*) cells containing WT or mutant *rcnR* genes on the *rcnR-PrcnA-lacZ* plasmid (pJI114)³⁶ were grown aerobically overnight at 37 °C in Luria-Bertani broth (LB) with chloramphenicol. These cultures were used to inoculate duplicate cultures in 2.0 mL LB with chloramphenicol in sterile culture tubes. These cultures were grown aerobically to an OD₆₀₀ ~0.3–0.7 and then placed on ice to arrest cell growth. The OD₆₀₀ of each culture was recorded prior to placing 200 μ L of culture into a 2.0 mL micro-centrifuge tube containing 800 μ L of the LacZ assay buffer.⁶³ For metal induction experiments, cells were treated in the same way, except that before the addition of cells to the duplicate cultures, a maximal concentration of metal that resulted in < 10% inhibition of growth was added to each culture (300 μ M Zn(OAc)₂, 130 μ M CoCl₂, or 700 μ M NiCl₂). Metal titration experiments were performed identically to the metal induction experiments. Metal was added to each individual culture with a range of 0 – 180 μ M CoCl₂ and 0 – 900 μ M NiCl₂. All experiments were performed in quadruplicate. Statistical significance was determined by performing a one-way ANOVA statistical analysis followed by Dunnett's test for multiple comparisons.

Fluorescence Anisotropy

5' TAMRA (5-carboxytetramethylrhodamine) labeled oligonucleotide GATTCTACTCCCCCCCAGTACCTG and non-labeled complementary strand CAGGTACTGGGGGGGAGTAGAATC were annealed by heating a 10 μ M solution of each strand in 10 mM HEPES, 150 mM NaCl, 5 mM EDTA, pH 7.0 to 95 °C and then cooling overnight. WT-, E63A-, and E63C-*EcRcnR* were prepared in 10 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1 mM TCEP, pH = 7.0 (FA buffer). A final DNA concentration of 10 nM was prepared in FA buffer in a 1 cm fluorescence cuvette (Firefly Scientific). *EcRcnR* was then titrated into the cuvette, and changes in anisotropy were measured using a QuantaMaster-7 2003 fluorimeter (Photon Technology International) fitted with polarizing filters (excitation: 543 nm, emission: 575 nm, averaging time: 10 s, independent replicates: 3). The data was fit using DynaFit⁶⁴ using a one-step model where one *EcRcnR* tetramer binds to one DNA molecule.

Computational studies

A model structure for tetrameric apo-*EcRcnR* has been previously calculated by homology modeling using all available crystal structures of CsoR (PDB codes: 2HH7, 3AAI, 4M1P, and 4ADZ), a copper-dependent transcription factor belonging to the same family of DNA-binding proteins as RcnR.⁵² To model the metal-bound forms of *EcRcnR*, the same procedure was repeated, using Modeller v9.12⁶⁵ with the same template structures and sequence alignments, and incorporating the additional experimental information available

from EXAFS analysis of the metal complexes of WT-*EcRcnR* to guide the calculations (see Table 3 for details), in analogy with a published protocol.⁶⁶ In particular, for each modeled structure, four metal ions were included in the calculation (one per subunit), in agreement with the prior characterization³⁵ as well as with the metal content analyses carried out in this study. The van der Waals parameters for Co(II), Ni(II), were derived from the Zn(II) parameters included in the CHARMM22 force field implemented in the Modeller v9.12 package by applying a scale factor calculated on the basis of the ionic radius of each metal ion. In all modeling calculations that included metal ions, constraints were imposed using a Gaussian-shaped energy potential for distances, angles and dihedrals in order to correctly position the metal ions (M) with respect to the experimentally identified ligated residues (see Table 3). Symmetry restraints were also applied in the calculation in order to obtain four symmetrically identical monomers. The best model was selected on the basis of the lowest value of the DOPE score in Modeller.⁶⁷ A loop optimization routine was used to refine the regions that showed higher than average energy as calculated using the DOPE score. The results of the PROCHECK analysis⁶⁸ for the final model were fully satisfactory (97.5% most favored regions, 2.5% additional allowed regions). The molecular (solvent-excluded) surfaces and graphics of *EcRcnR* model structures were calculated using the UCSF Chimera package (see supporting information for additional details).⁶⁹

RESULTS

Metal Site Structural Analysis

X-ray absorption spectroscopy (XAS) was used to examine the metal site structures of complexes of WT-*EcRcnR* and its Glu34 and Glu63 variants in order to elucidate whether these two residues are first coordination sphere ligands and if they are involved in distinguishing cognate (*i.e.*, Co(II) and Ni(II)) from non-cognate (*e.g.*, Zn(II) and Cu(I)) metals.

X-ray absorption near-edge structure (XANES) analysis was used to provide information regarding the coordination number and geometry of the metal centers. Metal ions with vacancies in the 3d orbitals, such as Co(II) and Ni(II), exhibit features below the edge energy that involve $1s \rightarrow 3d$ electronic transitions. This transition is symmetry-forbidden in centrosymmetric geometries, and thus the intensities of these features (peak areas) provide a measure of the centrosymmetric character of the metal site. Peak areas are smaller for more centrosymmetric geometries, such as octahedral and square-planar, and larger for non-centrosymmetric geometries, such as tetrahedral and square-pyramidal.^{70, 71} For geometries lacking one or more axial ligands (planar or pyramidal), a $1s \rightarrow 4p_z$ electronic transition is observed that can be used to distinguish between centrosymmetric geometries, *e.g.*, square-planar and octahedral, and between five-coordinate pyramidal and trigonal bipyramidal complexes.⁷¹ Being a d^{10} metal, Zn(II) lacks vacancies in the 3d manifold and thus no $1s \rightarrow 3d$ transition is possible. However, in this case, the intensity of the white line as well as number of transitions that follow the absorption edge are affected by coordination number and ligand type, allowing a qualitative analysis of the XANES spectra to be made for Zn(II).^{72, 73} XANES data for WT-*EcRcnR* and its Glu34 and Glu63 variants are summarized in Table 1 and Figure 2. (Additional fits useful in developing the final models are shown in

Supporting Information Tables S1 – S17. Enlargements of pre-edge features can be found in Figure S3).

At energies higher than the metal K-edge, extended X-ray absorption fine structure (EXAFS) analysis was used to provide metal-ligand distances with an accuracy of ~ 0.02 Å, information on the identity of the scattering atoms ($Z \pm 2$), and a second estimate on the coordination number ($\sim 20\%$ total number of ligands) that is complementary to the XANES analysis. The EXAFS spectra and best fits are summarized in Figures 3 – 6 and Table 1. Comprehensive fitting tables are included in the supporting information (Tables S1–17).

XAS of Co(II) complexes

A single pre-edge feature at ~ 7710 eV that is associated with a $1s \rightarrow 3d$ transition (Figure 2) is observed for the Co(II) complexes of both E34A- and E34C-*EcRcnR* proteins. The relatively small peak areas (Table 1) indicate that the Co(II) centers in these complexes have centrosymmetric ligand arrangements.^{71, 74} This, coupled with the lack of any other pre-edge feature, is consistent with an octahedral geometry in all cases. Thus, there is no obvious change in geometry associated with the variation of Glu34 or Glu63 as compared to WT-*EcRcnR*.^{75, 76} However, the XANES spectra for WT, E34A-, E34C-, E63A- and E63C-*EcRcnR* are non-overlapping, and thus indicative of some change in the Co(II) coordination sphere. These differences were addressed by EXAFS analysis.

EXAFS analysis of Co(II) E34A-*EcRcnR* (Table 1, Figure 3A) is consistent with a six-coordinate site, in agreement with the XANES analysis, and the best fit features two histidine imidazole ligands, two N/O-donors, a bromide ion from the buffer and a sulfur-donor that indicates coordination of Cys35. Incorporation of the Br^- ligand indicates the presence of an open coordination site, or solvent-exchangeable ligand, in the E34A-*EcRcnR* complex that does not exist in WT-*EcRcnR*. In addition to the replacement of a N/O-donor ligand with a Br^- , the E34A-*EcRcnR* site has a long Co-S bond at $2.54(4)$ Å, that is significantly altered compared to the Co-S distance in the Co(II) WT-*EcRcnR* complex (2.20 Å), and more closely resembles the WT-*EcRcnR* Ni(II)-SCys35 bond distance (2.63 Å). The resulting $[\text{Co}(\text{His})_2(\text{N/O})_2(\text{Cys})\text{Br}]$ complex is consistent with the role of Glu34 as a Co(II) ligand in WT-*EcRcnR*.

The EXAFS analysis of Co(II) E34C-*EcRcnR* is consistent with a six-coordinate complex, and therefore also consistent with the XANES analysis (Table 1, Figure 3B). The major change in the structure with respect to Co(II) WT-*EcRcnR* is the inclusion of two S-donor ligands (Cys34 and Cys35), one at a longer distance ($2.61(4)$ Å) and one at a shorter distance ($2.36(4)$ Å). The data are best fit by a model that includes two His imidazole ligands, and features a split N/O shell, where the difference in the M-N/O distances between

the two shells (0.15 Å) is at the calculated resolution of the data set ($r = \frac{\pi}{2 \times \Delta k} = 0.15$) and thus significant. Although it is not possible to identify which Cys residue is responsible for the long vs. short Co-S distances, the resulting $[\text{Co}(\text{His})_2(\text{N/O})_2(\text{Cys})_2]$ complex also supports the assignment of Glu34 as a Co(II) ligand in Co(II) WT-*EcRcnR*.

The EXAFS analysis of the Co(II) E63A-*EcRcnR* spectrum yields similar results to Co(II) E34A-*EcRcnR*. The EXAFS analysis is consistent with a six-coordinate geometry, in agreement with the XANES analysis. The best fit includes four N/O-donors, two of which can be modeled as histidine imidazole ligands, one long Cys35 S-donor, and one Br⁻, which replaces a N/O-donor ligand in the WT Co(II) site (Table 1 and Figure 3C). The Co-SCys35 distance (2.50(2) Å) is considerably longer than that observed for Co(II) WT-*EcRcnR*, and approaches the distance found in the Ni(II) WT-*EcRcnR* complex, as was the case for the Co(II) E34A-*EcRcnR* complex (*vide supra*). The resulting [Co(His)₂(N/O)₂(Cys)Br] complex differs from that found for Co(II) E34A-*EcRcnR* only by small differences in Co-L distances. Thus, the data are consistent with Glu63 acting as a Co(II) ligand in the Co(II) WT-*EcRcnR* complex.

The best fits of Co(II) E63A-*EcRcnR* in NaBr buffer suggest that the Co-Br distance is shorter than the Co-S distance. In an attempt to confirm this assignment, the experiment was repeated using a buffer containing 300 mM NaCl instead of NaBr (SI Tables S1 and S2; Figure S1 and S2). In this case, the data analysis is consistent with a [Co(His)₂(N/O)₂(Cys)Cl] complex, the major differences being substitution of Br⁻ by a Cl⁻ ligand. The latter is indistinguishable from an S-donor ligand and was therefore fit using S parameters, (see Table 1). However, the Co-S/Cl distances in the Cl⁻ complex are shorter (2.42 Å) and longer (2.66 Å) than the putative Co-Br⁻ and Co-S distances, and so no definitive assignment can be made. Regardless, the results are consistent with Glu63 coordination to Co(II) in WT-*EcRcnR*.

The results of EXAFS analysis for the Co(II) E63C-*EcRcnR* complex (Table 1, Figure 3D) also resemble those obtained for the corresponding E34C-*EcRcnR* complex, and is also consistent with the formation of a [Co(His)₂(N/O)₂(Cys)₂] complex with similar metric parameters. Thus, the results from both variants confirm the role of Glu63 as a Co(II) ligand in WT-*EcRcnR*.

XAS of Ni(II) complexes

The pre-edge features of the Ni(II) complexes of E34A- and E34C-*EcRcnR* are very different from each other, and distinct from the six-coordinate Ni(II) WT-*EcRcnR* complex. Both Glu34 variants exhibit a peak associated with a 1s → 3d transition located at ~8330 eV (Figure 2 and SI Figure S3). The peak area for the E34A-*EcRcnR* Ni(II) complex is very large (~ 10 × 10⁻² Å²), indicating a non-centrosymmetric geometry. Coupled with the absence of a peak associated with a 1s → 4p_z transition, the XANES is consistent with a seven-coordinate geometry, as suggested by the EXAFS analysis (*vide infra*). The peak area for the Ni(II) E34C-*EcRcnR* complex is ~ 5 × 10⁻² Å², and together with the presence of a shoulder associated with a 1s → 4p_z transition at ~8336 eV, is most consistent with a five-coordinate pyramidal complex.^{74, 75, 77}

Neither of the Ni(II) complexes of the E63A- nor E63C-*EcRcnR* variants have a visible 1s → 3d transition. As the peak is too small to be observed, it indicates that both complexes feature centrosymmetric Ni(II) environments. The E63A-*EcRcnR* Ni(II) complex does not feature any other visible pre-edge feature, consistent with an octahedral geometry. On the other hand, the spectrum of the Ni(II) complex of E63C-*EcRcnR* has a small shoulder that is

associated with a $1s \rightarrow 4p_z$ transition. This feature is not consistent with six-coordinate, trigonal-bipyramidal, or tetrahedral geometries. The presence of this shoulder is consistent with a five-coordinate pyramidal Ni(II) site, but the small $1s \rightarrow 3d$ transition is not. This situation could arise if, for example, the sample is a mixture of four-coordinate planar and six-coordinate Ni(II) sites, which would exhibit a feature associated with a $1s \rightarrow 4p_z$ transition from the planar component, but have a small $1s \rightarrow 3d$ peak area since that is characteristic of both geometries, and could average to five ligands in the EXAFS analysis (*vide infra*). Regardless of the exact coordination number, this variant shows a tendency toward loss of ligands in at least some of the Ni(II) sites, a trend that was also observed for the Ni(II) E34C-*EcRcnR* complex (*vide infra*).

The analysis of the EXAFS spectrum of the E34A-*EcRcnR* Ni(II) complex is best fit with a ligand environment that is composed of two histidine imidazole ligands, four additional N/O-donor ligands in two resolved shells, and a long ($\sim 2.61(1)$ Å) Cys35 S-donor. This yields a total of seven scattering atoms (Table 1 and Figure 4A). Although a six-coordinate complex would be within the error of coordination number determination by EXAFS analysis ($\pm 20\%$), a centrosymmetric arrangement of ligands is inconsistent with the large $1s \rightarrow 3d$ transition observed in the XANES spectrum. A seven-coordinate geometry is most consistent with both the EXAFS and XANES analyses, although a highly distorted six-coordinate complex cannot be ruled out. A six-coordinate fit of the EXAFS was attempted, but resulted in an unacceptably high σ^2 value for the first shell N-donor ligands. A seven-coordinate fit resulted in acceptable values for all parameters. In all other aspects, the E34A-*EcRcnR* Ni(II) site is essentially unaltered from Ni(II) WT-*EcRcnR* (Table 1). In addition, the complex does not bind exogenous Br^- , as expected for a 'loss of ligand' variant. Replacement of Glu34 by a water molecule in preference to Br^- cannot be ruled out in principle, though it would be inconsistent with the incorporation of Br^- in Co(II) complexes of E34A- and E63A-*EcRcnR* (*vide supra*), and with the Ni(II) complex of E63A-*EcRcnR* (*vide infra*). Moreover, the substitution of Glu34 by water would modify the charge on the metal center, and would be expected to significantly alter the XANES spectrum, in contrast with the observation.

In contrast to the data obtained for the Ni(II) E34A-*EcRcnR* complex, the EXAFS data from Ni(II) E34C-*EcRcnR* indicates a decrease in the coordination number from six to five (Table 1, Figure 4B). This change in coordination is consistent with the XANES result, which indicated a five-coordinate pyramidal geometry. In addition to a change in coordination number, the EXAFS analysis reveals that the Ni(II) center in E34C-*EcRcnR* has two S-donor ligands (Cys34 and Cys35), with one long, WT-like, Ni-S distance at $2.56(3)$ Å, and a shorter Ni-S bond at $2.29(2)$ Å. The resulting $[\text{Ni}(\text{His})_2(\text{N/O})(\text{Cys})_2]$ complex minimally results from substitution of Glu34 by Cys and loss of an N/O-donor ligand. This could be interpreted as resulting from the substitution of a bidentate carboxylate by Cys. However, neither the E34A- nor the E34C-*EcRcnR* Ni(II) sites give unambiguous results for simple 'loss of ligand' or 'ligand substitution' variations.

The EXAFS data for the Ni(II) E63A-*EcRcnR* site is best fit by a six-coordinate model featuring four N/O-donors, two of which are imidazole ligands (Tables 1, and Figure 4C). The coordination sphere is completed by a bromide ion from the buffer and a Ni-SCys35

bond (2.50(2) Å) that is shorter than in the WT-*EcRcnR* Ni(II) complex (2.63(2) Å). This [Ni(His)₂(N/O)₂(Cys)Br] complex results from replacement of Glu63 by Br⁻ in Ni(II) WT-*EcRcnR*, similarly to what is observed in the Co(II) complex of E63A-*EcRcnR*, and consistent with Glu63 acting as a ligand to Ni(II), as well as to Co(II), in WT-*EcRcnR*.

As was the case for E34C-*EcRcnR*, the analysis of the EXAFS spectrum of the Ni(II) complex of E63C-*EcRcnR* (Figure 4D) is consistent with coordination of a second S-donor and a reduced coordination number. The resulting five-coordinate [Ni(His)₂(N/O)(Cys)₂] complex involves the loss of two N/O-donor ligands relative to the WT-*EcRcnR* structure, one of which is replaced by the Cys63 S-donor, and resulting in one long (2.55(4) Å) and one short (2.30(3) Å) Ni-S distance. The large number of structural changes makes it difficult to unambiguously determine which S-donor belongs to which cysteine residue. But, taken together, the results of both Glu63 variants are clearly consistent with ligation of Glu63 in the Ni(II) WT-*EcRcnR* complex.

XAS of Zn(II) complexes

The ratio of the normalized intensities of the two post-edge XANES features for the Zn(II) complexes of WT-*EcRcnR* and all variants are ~1.3, which is consistent with either four- or five-coordinate Zn(II) sites.⁷¹ XANES spectra of all Zn(II) complexes (Figure 2) exhibit features at 9666.6 and 9671.0 eV that are associated with 1s → 4p transitions, and are consistent with a Zn(II) center coordinated by a combination of N/O-donors together with heavier scattering donor-atom ligands, such as sulfur or bromide.⁷²

The results of the analysis of the EXAFS data for Zn(II) complexes of E34A- and E34C-*EcRcnR* (Table 1, Figure 5A and B) are generally consistent with the qualitative XANES analysis, and indicate that the Zn(II) sites in these variants are essentially identical, and very similar to the Zn(II) complex in WT-*EcRcnR*. The best fits in all cases include two His ligands, one N/O-donor ligand, one Br⁻ ligand and one S-donor. The resulting five-coordinate [Zn(His)₂(N/O)(Cys)Br] complexes demonstrate that Glu34 is not a ligand for Zn(II) in WT-*EcRcnR*.

The EXAFS analysis of Zn(II) E63A-*EcRcnR* is indicative of a site composed of three N/O-donors, one S-donor from Cys35 and a bromide from the buffer (Table 1), and is consistent with expectations based on the XANES analysis (*vide supra*). However, unlike the Zn(II) complex of WT-*EcRcnR*, where EXAFS data can be modeled as a [Zn(His)₂(N/O)(Cys)Br] complex (Table 1), the best models for the Zn(II) sites in E63A- and E63C-*EcRcnR* contain three histidine imidazole ligands, [Zn(His)₃(Cys)Br] (Table 1, Figure 5C and D). Attempts to fit the EXAFS spectrum for the E63A Zn(II) site using two imidazole ligands and 1 N/O ligand, in analogy with the WT case, result in slightly lower values of %R and reduced χ^2 , but also yield a very large value of σ^2 for the Zn-SCys35 bond, although the Zn-S distance is unchanged between the two models (Table 1). A similar fit, involving two imidazole ligands and 1 N/O ligand, could be obtained for the Zn(II) complex of E63C-*EcRcnR*, but the values of %R and reduced χ^2 were higher.

The Zn(II) sites in E63A- and E63C-*EcRcnR* show subtle structural changes (possible increased imidazole ligation) that are not consistent with Glu63 being a ligand in the WT-

RcnR Zn(II) complex, but might suggest a role for Glu63 in ordering the ligands in Zn(II) site.

Electronic factors in the structures of the Ni(II) sites in EcRcnR variants

Susceptibility measurements using the Evans NMR method were made on samples of WT- and E63C-*EcRcnR* Ni(II) complexes, and the parameters obtained from the spectra (SI Figure S4) and calculated values of are shown in Table 2. The results show that WT-*EcRcnR* contains a paramagnetic Ni(II) center with $\mu = 3.4$ BM, which is in the range expected for high-spin Ni(II) complexes (2.8 – 4.0 BM),⁷⁷ and consistent with a six-coordinate $S = 1$ Ni(II) center. The sample of E63C-*EcRcnR* has greatly reduced paramagnetism that would correspond to a Ni(II) center with $\mu = 1.5$ BM, which is too low to result from a high-spin Ni(II) complex, and indicates that most of the Ni(II) in the sample is diamagnetic (low-spin). The ratio of the molar susceptibilities of WT- to E63C-*EcRcnR* Ni(II) complexes indicates that the solution of the variant is ~20% as paramagnetic as the WT-*EcRcnR* sample, suggesting the presence of a paramagnetic impurity that might arise from an equilibrium of five- and six-coordinate species, with the six-coordinate complex serving to provide the paramagnetism and moderate the XANES features normally associated with a five-coordinate pyramidal complex (*vide supra*).

Functional Analysis

Previous studies³⁵ revealed that expression of P_{rcnA} occurs only in the presence of Ni(II) and Co(II), but not upon addition of Mn(II), Fe(II), Cu(II), Zn(II), and Cd(II). The effects of the Glu → Ala and Glu → Cys variations on the metal responsiveness of *EcRcnR* were tested using a LacZ reporter assay (Figure 6, Table S18). Statistically significant differences are indicated with stars in Figure 6. Both E34A- and E34C-*EcRcnR* had metal ion responses that are indistinguishable from WT-*EcRcnR*, demonstrating that ligation of Glu34 is not important to metal-induced transcription, regardless of the structural role identified by XAS analysis. On the other hand, while the E63A-*EcRcnR* variant also shows a WT-like response to Co(II)-binding, derepression is reduced by ~50% in response to Ni(II)-binding. The E63C-*EcRcnR* variant also shows a ~50% reduction in response to Ni(II), but also displays a 25% decrease in response to Co(II). All four variants remain unresponsive to Zn(II)-binding, indicating that metal selectivity has not been impaired by the Glu34 and Glu63 mutations. These results suggest that while mutation of both Glu34 and Glu63 affect the metal site structure, only mutation of Glu63 significantly affects the metal-responsiveness of RcnR in the cell. The data also indicate that while Glu63 may be involved in ordering the Zn(II) site (*vide supra*), this ordering imparts no obvious change to WT-*EcRcnR*'s lack of response to binding Zn(II).

The LacZ reporter assays were performed at metal concentrations that produced maximal activity without being detrimental for cell growth. Thus, even though the Glu34 mutations did not affect the functional response at maximal metal concentrations, metal titrations were performed to see if the mutations affected the LacZ activity at lower metal concentrations. In all cases, there was no difference between the response to metal of WT-*EcRcnR* and that of the two Glu34 variants at concentrations ranging from 0 μ M to 900 μ M (SI Figure S5). These studies also reveal that WT-*EcRcnR* begins to respond to Ni(II) at ~300 μ M NiCl₂,

similar to a previously reported value,³⁶ and to Co(II) at ~10 μ M CoCl₂. This response is unchanged for both E34A- and E34C-*EcRcnR*.

Protein structure and DNA Binding

No changes in the quaternary structures of the Glu variants with respect to WT-*RcnR* was observed by size exclusion chromatography (SI, Figure S6). Both Glu63 variants showed diminished transcriptional response to the addition of Ni(II) and Co(II). In order to test if this is due to increased DNA-binding affinity, fluorescence anisotropy measurements were performed. 5-carboxytetramethylrhodamine (TAMRA) labeled Site-2 DNA, containing one of two recognition sequences,⁴⁴ was titrated with WT-, E63A-, and E63C-*EcRcnR*. The apparent K_{DNA} of WT-*EcRcnR* is 13.64 ± 3.9 nM with no significant change in the apparent K_{DNA} of either mutant (20.75 ± 4.9 nM for E63A-*EcRcnR* and 17.02 ± 6.9 nM for E63C-*EcRcnR*) (SI, Figure S7). This indicates that the E63A- and E63C-*EcRcnR* variants did not alter the DNA binding affinity of the apo-*RcnR* proteins compared to WT, and is compelling evidence that they are correctly folded. The decrease in metal-induced transcriptional responses is not a consequence of increased DNA-binding affinity of the apo-protein.

Computational models of metal bound WT-*EcRcnR*

In view of the data presented above regarding the role of Glu34 and Glu63 in metal binding, the initial computational models used to identify these residues as potential metal ligands⁵² were refined. All three modeled metal sites feature Cys35 thiolate as a common ligand, because this is the only Cys residue in the protein and S-donor ligation is observed in the EXAFS spectra of all the metal complexes.^{47, 48} In all cases, the metal ions bind at a dimer interface that connects the N-terminal portion of helix α_2 of one monomer to the C-terminal portion of the same helix from the adjacent monomer. The models show that one monomer provides most of the potential metal ligands, namely the N-terminal amine, His3, His60, His64, and His67, whereas Glu34 and Cys35 are derived from the other monomer at the dimer interface. This situation is analogous to what observed in the crystal structures of Cu(I)-CsoR.³⁹

All constraints are listed in the form: mean value $\pm$ 1 standard deviation. When not differently indicated, distance restraints have a standard deviation of ± 0.05 Å with the respect to the equilibrium distances. No restraints were added to constrain the coordination geometry around the metal ions.

Co(II) WT-*EcRcnR*—The EXAFS analysis presented above provides evidence for Glu34 and Glu63 as ligands to the Co(II) site in WT-*EcRcnR*. These results, along with prior identifications of additional Co(II) ligands,^{35, 47, 48} lead to a proposed six-coordinate (His)₂(Glu)₂(Cys)Ser2-NH₂ site for WT-*EcRcnR*. The His ligands involved are His3 and His64, the Glu ligands are Glu34, and Glu63, the Cys is Cys35, and Ser2-NH₂ represents the N-terminal amine ligand generated upon cleavage of Met1.³⁵ His60 was also proposed to be involved in the Co(II) binding site as a second coordination sphere residue that is H-bonded to one of the residues directly bound to the metal ion.⁴⁸ The calculation was conducted by imposing distance and angle restraints to each of the four Co(II) ions in order to bind them to Ser2-NH₂, His3, Glu63, and His64 from one chain and Glu34 and Cys35 from the second

chain comprising each dimeric unit (Table 3). A preliminary calculation showed a small distortion in the His3 aromatic ring if the restraints are imposed on Ne, and so N6 was used to bind Co(II). The result of the first modeling scheme (named Co-1) are reported in Figure 7A. The coordination geometry around the modeled Co(II) binding site is compatible with the distorted octahedral complex found by XAS analysis. The bond distances are in good agreement with those determined experimentally (Table 1 and SI Table 3). The only Co(II) binding residue in the vicinity of His60, which is known to contribute to this binding site without interacting directly with the metal ion, is Cys35, and therefore a new model named Co-2 was prepared with the same constraints as Co-1 plus an additional distance restraint of 3.00 ± 0.10 Å between Cys35 and His60(Ne) to model a His60 – Cys35 H-bond. The results of the Co-2 calculation are reported in Figure 7B. The Co(II) binding site is unchanged with respect to the Co-1 model and His60 is found in a good orientation to perform a H-bond with Cys35.

Ni(II) WT-EcRcnR—The analysis of the XAS data for the Ni(II) complex with EcRcnR is consistent with a (N/O)₅S center with one or two histidine ligands and contributions from one cysteine ligand. One ligand has been experimentally identified to be the Ser2 N-terminal amine, while site-directed mutagenesis was used to identify other metal ligand residues as Cys35, Glu63, and His64. His3 was specifically excluded as a Ni(II) ligand. The aim of our modeling calculation was to identify other putative Ni(II) ligands in order to complete the coordination sphere of the ion. In the first modeling scheme (named Ni-1) each Ni(II) ion was constrained to coordinate Ser2-NH₂, Glu63, and His64 from one chain together with Cys35 from the second chain of each dimeric unit. In this model, the metal ion is coordinated in a distorted four-coordinate planar geometry with Ser2-NH₂ trans to His64 and Cys35 trans to Glu63 (Figure 8A).

The constraints used in the Ni-1 calculation lead to a model that does not fill the Ni(II) coordination sphere as determined by the XAS data (Figure 8A). This consideration prompted us to prepare a second modeling scheme (named Ni-2) in which Glu63 was imposed as a bi-dentate ligand. This still would leave one empty position, which is filled by the carbonyl oxygen of Ser2. The results of the Ni-2 calculation are reported in Figure 8B. The Ni(II) ion is coordinated in a highly distorted octahedral geometry, consistent with the XANES analysis (*vide supra*). The calculated bond distances in this case are also in agreement with those determined experimentally by EXAFS analysis (Table 1 and Table 3).

Zn(II) WT-EcRcnR—The fitting of the XAS data for the Zn(II) complex with EcRcnR is consistent with a (N/O)₂SBr or with a (N/O)₃SBr center, with two histidine ligands and contributions from one cysteine sulfur and one bromide derived from the buffer (Figure 9). The histidine and the cysteine residues appear to be His67 and Cys35, while His3 and the N-terminal amine are not involved in Zn(II) binding. Thus, the aim of our modeling calculation was to identify other putative Zn(II) ligands in order to complete the coordination sphere of the ion and give hints on the coordination number. The present study also indicated that neither Glu34 nor Glu63 are ligands to Zn(II), with Glu63 having a marginal role in ordering the Zn(II) site (Figures 9 and S8). Multiple Zn(II) models were attempted and are discussed in detail in the supporting information. Among these, only model Zn-2b accurately

reproduces the experimentally determined coordination number and ligand identity. In this model, Cys35, His64 and His67 are included as Zn(II) ligands in addition to one Br⁻ from solvent (Figure 9). The bond distances are in good agreement with those determined experimentally (Table 1). This model suggests that His64 completes the coordination sphere as the second His ligand in addition to His67. A previous study, involving mutation of His64 to Cys, resulted in a six-coordinate [Zn(His)₃(Cys)₂Br] complex,⁴⁸ supporting His64 as a ligand. In the model, it appears that a putative and indirect role of Glu63 involves the formation of a second-shell H-bond between the carboxylate group of this residue and Cys35-Sγ. A similar interaction, involving a metal-bound cysteine thiolate and a second shell residue acting as an H-bond donor has been reported in the case of the copper center in plastocyanin.⁷⁸

DISCUSSION

RcnR is one of only a few metallosensors for which high-coordination number sensing sites have been identified (*e.g.*, NmtR, MntR).^{10, 79} Establishing the full primary coordination sphere of metals bound to *Ec*RcnR is important for understanding its cellular function as a Ni- and Co-responsive metal sensor protein. Knowledge of the complete set of ligands will also help to explain the unusual features of Ni-coordination, in particular a six-coordinate, high-spin (*S* = 1) complex that features Cys thiolate coordination at 2.65 Å, and the basis by which *Ec*RcnR senses both Ni(II) and Co(II) with different sets of ligands.

Metal-site structures

The results shown above associate Glu34 and Glu63 with cognate metal binding by demonstrating that altering either glutamate residue to alanine or cysteine causes significant changes to the cognate metal site structures, but not to the non-cognate Zn(II) site structure. In the case of Co(II), the structural changes observed in the glutamate variants are the ones anticipated for loss of a ligand (E34A; E63A) and a ligand substitution (E34C; E63C), namely loss of an N/O-donor ligand and incorporation of Br⁻ from the buffer and substitution of an O/N-donor ligand by the Cys34 S-donor in the mutant protein, respectively. Thus, Glu34 and Glu63 are clearly established as Co(II) ligands in WT-*Ec*RcnR. This completes the coordination sphere for Co(II), which has now been shown to bind the N-terminus, His3, Glu34, Cys35, Glu63, and His64.^{47, 48} It is worth noting that the ligation of Co(II) by *Ec*RcnR resembles a tris-bidentate chelate complex in that it involves coordination of neighboring pairs of residues the His3/N-terminal amine, Glu34/Cys35, and Glu63/His64.

The nature of the structural changes observed for the Ni(II) complexes of E34A- and E34C-*Ec*RcnR are more ambiguous. Alteration of Glu34 to alanine shows formation of what is best described as a seven-coordinate complex with an (N/O)₆S ligand donor-atom set. One possible explanation is that Glu34 is a Ni(II) ligand, and the vacancy created in E34A-*Ec*RcnR is filled either by a water molecule in preference to bromide, or by causing Glu63 to become bidentate to fill the vacancy. The combination of these two changes would result in a seven-coordinate complex, with Glu63 becoming bidentate in part to compensate for the increased positive charge on the metal. Another possibility is that Glu34 is not a ligand, but

that modification of Glu34 perturbs the position of Glu63, possibly via alteration of an H-bonding network, such that Glu63 becomes bidentate, resulting in a complex that is seven-coordinate in terms of donor-atoms, but involves coordination of only six protein residues. Modeling of the WT-*EcRcnR* Ni site does indicate the possibility that Glu63 may bind in a bidentate fashion (*vide supra*). A similar situation was observed for Ni(II) H3E-*EcRcnR* mutation, which also adopted a 7-coordinate site that was hypothesized to result from bidentate glutamate binding.⁴⁷ Further, conversion of monodentate carboxylate ligands to bidentate carboxylate ligands has been observed upon mutation of Asp8 to Met in a Mn(II) site in the manganese metallosensor, MntR,² an alteration that does not affect the Mn(II) response.

The results for the Ni(II) complex of the “ligand substitution” variant E34C-*EcRcnR* do not directly help resolve the role of Glu34 in coordination of Ni(II) by WT-*EcRcnR*. This variant results in multiple structural changes in the Ni(II) site, namely the loss of a N/O-donor ligand and substitution of another by Cys34, yielding a five-coordinate complex with two S-donor ligands ((N/O)₃S₂). This change is not consistent with a single ligand substitution, and therefore obscures the role of Glu34 as a ligand by this criterion. However, it is possible that the ligand substituted is a bidentate Glu34, which would yield this result.

One likely reason for the large structural perturbation of the Ni(II) site in the Glu → Cys variants comes from the studies of the Ni(II) complexes of the Glu63 variants. The XAS results for the E63A-*RcnR* Ni(II) complex show the expected results for a loss of ligand mutation where one N/O-donor replaced by a bromide ion from the buffer, and thus clearly establishes Glu63 as a Ni ligand. The structure of the of E63C-*EcRcnR* Ni(II) complex is similar to the structure found for Ni(II) E34C-*EcRcnR*, namely a 5-coordinate structure containing two S-donor ligands that involves the loss of one N/O-donor as well as coordination of the second S-donor (Cys63), resulting in a five-coordinate complex with (N/O)₃S₂-donor ligands. The change in coordination number/geometry that accompanied coordination of Cys63 suggested a change in electronic structure, possibly involving a conversion to a low-spin configuration that is associated with lower coordination numbers. This is supported by susceptibility measurements using the Evans NMR method, in which WT-*EcRcnR* contains a paramagnetic Ni(II) center, consistent with the six-coordinate structure, but E63C-*EcRcnR* is ~80% diamagnetic, and thus largely consistent with a low-spin five-coordinate complex.

All Ni(II) complexes with an octahedral geometry are high-spin and paramagnetic with $S = 1$. In contrast, all four-coordinate planar complexes of Ni(II) are low-spin, with $S = 0$. Five-coordinate pyramidal complexes are roughly evenly divided between high- and low-spin electronic configurations, depending on the relative strength of the apical and basal ligand fields. The role of cysteine thiolate ligands in determining the spin-state of Ni(II) has been explored using a mutagenic approach in NiSOD, where it was concluded that both cysteine ligands present in the NiSOD active site were required to maintain a low-spin configuration.⁸⁰ Loss of either Cys ligand led to conversion to a high-spin complex with no Cys ligation. In E34C-*EcRcnR*, the addition of a second S-donor ligand results in Ni(II) complexes that undergo a spin conversion to a low-spin configuration and lose a N/O-donor ligand (the reverse of the situation described above for NiSOD). This may also be relevant to

the Ni(II) site structure in InrS, a related transcriptional regulator in the RcnR family that contains a low-spin, four-coordinate planar site that is supported by two Cys thiolate-donors.⁵⁰ The change in structure that occurs with the spin-state change also points out a limitation in our mutation strategy in that the ligand substitution of an N/O-donor for an S-donor, which is easily detected by EXAFS analysis, can have unintended structural consequences as a result of a change in the spin-state of Ni(II).

The six-coordinate high-spin monothiolate Ni(II) complex formed with WT-RcnR is very unusual. With respect to metal coordination by WT-RcnR, the results presented above indicate a Ni(II) site in WT-*Ec*RcnR features coordination by the N-terminus, Cys35, Glu63, His64, and two other N/O-donors, one of which might be Glu34. The similar results obtained for the Ni(II) complexes of E34C-RcnR and E63C-RcnR are consistent with similar roles as ligands for the two glutamate residues, although this role for Glu34 is still ambiguous because the results of XAS analysis for E34A-RcnR are not conclusive. It has been previously noted that few high-spin Ni(II) thiolate complexes have been characterized,³⁵ and the existing examples feature very long (> 2.5 Å) Ni-S distances, like that of the WT-*Ec*RcnR Ni(II) complex, and are generally supported by bidentate coordination, presumably to prevent facile substitution of the weak S-donor ligands.^{81, 82} This supporting role may be the function of the neighboring Glu34 residue, which could act to support the high-spin six-coordinate monothiolate Ni(II) complex that utilizes the only Cys residue in the protein. For Ni, the computational models suggest that a similar structure to Co, mentioned above, is obtained, where coordination by the Ser2 amide O atom replaces the His3 imidazole, forming a true five-membered chelate ring with the terminal Ser-NH₂.

Implications for Function

The present and previous studies of RcnR mutants have revealed discrepancies between functional responses to metal compared to effects on metal coordination. The available structure/function data from XAS and LacZ studies for the cognate metal complexes of *Ec*RcnR are summarized in Table 4, which indicates whether a residue is or is not a metal ligand (XAS), and whether the residue contributes to a transcriptional response to metal binding (LacZ). There are three main outcomes of these sets of data. First, XAS demonstrates that Co and Ni ions bind different, but overlapping, ligand sets. Second, LacZ assays reveal that there are residues important for metal coordination that when mutated show little effect on the transcriptional response for at least one metal, and *vice versa*. A response to metal in the LacZ assay by an RcnR mutant requires a metal-binding affinity tight enough to detect the metal *in vivo*, as well as the capability of metal-binding to regulate DNA-binding allosterically. The consequence of a weakened affinity mutant is greater metal accumulation because induction of efflux by RcnA is impaired, but the feedback does not extrapolate with ever weakening affinities, as recently shown for the Ni-responsive RcnR homolog, InrS.⁸³ Thus, mutants with altered metal coordination geometry as determined by XAS that maintain a normal transcriptional response are predicted to have a metal-binding affinity within 20–30 fold of the wild-type protein and be capable of transmitting metal-binding site occupancy to DNA-binding residues.

Second coordination sphere interactions important in the allosteric response to metal binding can contribute structural features that cannot be directly probed by XAS. These interactions are known to play important roles in generating metal-specific responses in metalloregulators. Mutation of these residues will yield wild-type like XAS metal-site structures but be non-responsive to one or more metals in the LacZ assay. For example, mutation of a second sphere Glu residue (E81) in the Cu(I)-sensing CsoR protein has negligible effect on Cu(I)-affinity but a large effect on the allosteric response.⁸⁴ Furthermore, in the MerR family of transcriptional regulators, it has been shown that the metal discrimination *in vitro* between monovalent and divalent cations, and between different pools of monovalent and divalent cations, is conferred by second coordination sphere interactions and not through the primary metal coordination sphere.^{9, 84}

Despite the obvious structural roles played by Glu34 and Glu63 in coordination of the cognate metals, neither residue is critical to the allosteric response to specific metal binding that facilitates DNA release. Both Glu34 variants retain full metal-responsive transcriptional regulation to both cognate metals, and both Glu63 variants retain substantial, although sometimes partially diminished, responses. The lack of a correlation between ligation of specific Glu residues and transcriptional response in *EcRcnR* indicates that not every ligand in the primary coordination sphere plays a critical role in the allosteric response involved in regulating transcription. This contrasts with the intensively studied Zn(II)-responsive CzrA protein, in which mutation of any of the four metal-ligands affects function in some way.⁸⁵ Thus, identification of a residue as a metal ligand does not infer functional importance, and identification of ligands by functional assay alone is also limited. In *EcRcnR*, where the ligand selection of the two cognate metals is different, Glu34 and Glu63 may provide a basis for differential response to these two metals.

Mechanistic considerations

A critical requirement for the allosteric response of *EcRcnR* appears to be metal-coordination of N-terminus. Binding of the N-terminal amine and, in the case of Co(II), the His3 side chain imidazole, may be involved in positioning the N-terminus of the α 1-helix over the metal-binding site, in analogy with studies of *Geobacillus thermodenitrificans* CsoR.⁴⁹ The allosteric response would not occur for non-cognate metals, since they do not bind these residues. This situation is similar to what has been recently determined for the Ni(II)/Co(II) responsive membrane-bound periplasmic regulator CnrX.^{46, 86–88} Mutational studies on CnrX indicate that binding of Met123 pulls the protein into a signal propagating active conformation. Non-cognate metals do not bind this residue and thus cannot facilitate this conformational change.

Remarkably, these studies show that the coordination number (and even spin-state!) of the cognate metal ion is relatively unimportant to the allosteric response in *EcRcnR*. However, coordination number is important for discriminating cognate from non-cognate metals in *EcRcnR*.^{47, 48} Resolving the details of the link between metal coordination and transcriptional response to metal binding will require determination of metal-affinities of individual mutants for both cognate and non-cognate metals and the corresponding changes

in DNA-binding affinity that are linked to the transcriptional response monitored by the LacZ assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

RcnR	resistance to cobalt and nickel regulator
Amp	ampicillin
cam	chloramphenicol
CsoR	copper-sensitive operon repressor
XAS	X-ray absorption spectroscopy
XANES	X-ray absorption near-edge spectroscopy
EXAFS	extended X-ray absorption fine structure
InrS	internal nickel-responsive sensor
ICP-OES	inductively coupled plasma optical emission spectroscopy
IPTG	isopropyl β -D-1-thiogalactopyranoside
NikR	nickel-responsive regulator of the <i>nik</i> operon
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
SlyD	sensitive to lysis D
HypA	hydrogenase protein A

RicR	regulated in copper repressor
CstR	CsoR-like sulfur transferase repressor
FrmR	formaldehyde regulator

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Synopsis

E. coli RcnR is a Ni(II)- and Co(II)-responsive transcriptional regulator involved in metal efflux. This work reports structure/function investigations of metal site glutamate protein variants. While the glutamate residues play a structural role in binding the cognate metals, they are not required for function. The flexibility of the metal site structures is striking, and includes spin-state changes in Ni(II) complexes that cause a conversion from high- to low-spin, but are unimportant to allosteric response.

Figure 1. (A) Ribbon schemes and solvent-excluded surfaces of the tetrameric apo-*EcRcnR* model structure.⁵² (B) Details of the proposed metal binding region at the interface between two monomer chains (α , orange and β , green). Atoms are colored according to atom type.

Figure 2. K-edge XANES spectral overlays for metal complexes of WT-*EcRcnR* and Glu variants in buffer with 20 mM HEPES, 300 mM NaBr, 1 mM TCEP and 10% glycerol (pH 7.0). The inset shows the pre-edge region for clarity.

Figure 3.

K-Edge EXAFS spectra for the Co(II) complexes of (A) WT-, (B) E34A-, (C) E34C-, (D) E63A-, and (E) E63C-*EcRcnR* in buffer containing 20 mM HEPES, 300 mM NaBr, 1 mM TCEP and 10% glycerol (pH 7.0). Left: Fourier-transformed EXAFS data (colored lines) and fits (black lines). Right: unfiltered k^3 -weighted EXAFS spectra and fits. Metric details for the fits shown are in bold face type in Table 1.

Figure 4.

K-Edge EXAFS spectra for the Ni(II) complexes of (A) WT-, (B) E34A-, (C) E34C-, (D) E63A-, and (E) E63C-*EcRcnR* in buffer containing 20 mM HEPES, 300 mM NaBr, 1 mM TCEP and 10% glycerol (pH 7.0). Left: Fourier-transformed EXAFS data (colored lines) and fits (black lines). Right: unfiltered k^3 -weighted EXAFS spectra and fits. Metric details for the fits shown are in bold face type in Table 1.

Figure 5.

K-Edge EXAFS spectra for the Zn(II) complexes of (A) WT-, (B) E34A-, (C) E34C-, (D) E63A-, and (E) E63C-*EcRcnR* in buffer containing 20 mM HEPES, 300 mM NaBr, 1 mM TCEP and 10% glycerol (pH 7.0). Left: Fourier-transformed EXAFS data (colored lines) and fits (black lines). Right: unfiltered k^3 -weighted EXAFS spectra and fits. Metric details for the fits shown are in bold face type in Table 1.

Figure 6. Results of LacZ transcription reporter assays showing the effects of the E34A-, E34C-, E63A-, and E63C-*EcRcnR* mutations on the expression of P_{rcnA} in response to metal ions. A statistically significant difference from wild-type response is indicated by a * ($p < 0.01$, one-way ANOVA followed by Dunnett's test for multiple comparisons).

Figure 7.

Panels **A** and **B** report the results for Co-1, Co-2 modeling, respectively. The *EcRcnR* backbone is reported as ribbons colored by polypeptide chain, with chain α in orange and chain β in green. Putative metal binding residues are reported as sticks colored accordingly to atom types (labeled with single letter amino acid code, residue number, and polypeptide chain identification α or β). The Co(II) ion is shown as an orange sphere. The reported distances are in Angstroms.

Figure 8.

Panels **A** and **B** report the results for Ni-1 and Ni-2 modeling, respectively. The *EcRcnR* backbone is reported as ribbons colored accordingly to the protein chain, as in Figure 7. Putative metal binding residues are reported as sticks colored accordingly to atom types. The Ni(II) ions are shown as green spheres. The reported distances are in Angstroms.

Figure 9.

Results of the Zn-2b modeling (see supporting information). The *EcRcnR* backbone is reported as ribbons colored accordingly to the protein chain, as in Figure 7. Putative metal binding residues are reported as sticks colored according to atom types. The Zn(II) and Br⁻ ions are shown as brown and dark red spheres, respectively. The reported distances are in Angstroms.

Table 1
XAS Analysis for Metal Complexes of WT-, Glu34-, and Glu63-RcnR Variants in Buffer with 300 mM NaBr ^a

XANES Analysis					EXAFS Analysis				
Sample	K-edge energy (eV)	1s → 3d peak area (×10 ² eV)	1s → 4p _z observed	Coord #	Shell	r (Å)	σ ² (×10 ⁻³ Å ²)	E ₀ (eV)	%R
WT-RcnR									
Co(II)	7720.2	6(1)	No	6	1 N/O	1.97(4)	2(3)	-2(2)	4.1
					2 N/O	2.20(4)	0(2)		
					1 S	2.20(6)	5(6)		
					1 Im 0°	2.09(4)	4(3)		
Ni(II)	8342.7	2(1)	No	6	1 Im 10°	2.09(3)	2(3)		
					2 N/O	2.07(2)	2(2)	4(1)	3.8
					1 N/O	2.21(3)	3(4)		
					1 S	2.63(2)	6(2)		
Zn(II)	9663.0	NA	NA	4/5	1 Im 0°	2.07(3)	3(2)		
					1 Im 10°	2.02(6)	6(5)		
					1 N/O	1.94(3)	7(4)	-4(1)	1.2
					1 S	2.30(2)	7(3)		
					1 Br	2.41(1)	5(1)		
					1 Im 0°	1.99(2)	0(3)		
					1 Im 10°	2.10(2)	1(2)		
					1 S	2.30(2)	5(2)	-5(1)	1.5
					1 Br	2.41(1)	5(1)		
					1 Im 0°	2.07(2)	2(1)		
					1 Im 0°	1.95(2)	0(2)		
					E34A-RcnR				
Co(II)	7720.6	6(1)	No	6	1 N/O	2.02(1)	5(1)	2(2)	1.7
					1 N/O	2.16(1)	6(1)		
					1 S	2.54(4)	1(4)		
					1 Br	2.50(3)	3(3)		
					1 Im 0°	2.02(7)	6(9)		
					1 Im 0°	2.16(8)	6(11)		

XANES Analysis						EXAFS Analysis			
Sample	K-edge energy (eV)	1s → 3d peak area (×10 ² eV)	1s → 4p _z observed	Coord #	Shell	r (Å)	σ ² (×10 ⁻³ Å ²)	E ₀ (eV)	%R
Ni(II)	8343.9	10(1)	No	7	1 N/O	2.04(1)	13(1)	7(1)	3.2
					2 N/O	2.19(2)	9(2)		
					1 S	2.60(2)	3(2)		
					1 Im 0°	1.94(2)	9(1)		
					1 Im 0°	2.10(2)	3(2)		
					2 N/O	2.07(1)	9(1)	7(1)	3.2
Zn(II)	9663.0	NA	NA	4/5	2 N/O	2.22(2)	7(2)		
					1 S	2.61(1)	3(1)		
					1 Im 0°	1.94(2)	8(1)		
					1 Im 0°	2.11(2)	7(1)		
					1 N/O	1.98(4)	7(6)	-3(1)	1.9
					1 S	2.28(3)	9(4)		
Co(II)	7720.4	6(1)	No	6	1 Br	2.43(1)	6(1)		
					1 Im 0°	2.11(2)	2(2)		
					1 Im 0°	1.98(2)	1(2)		
					1 S	2.27(3)	8(3)	-4(2)	2.9
					1 Br	2.42(1)	6(1)		
					1 Im	1.96(1)	2(1)		
Ni(II)	8344.3	5(2)	Yes	5	1 Im	2.09(2)	2(2)		
					E34C-RenR				
					1 N/O	1.99(3)	1(3)	3(2)	1.5
					1 N/O	2.14(2)	4(2)		
					1 S	2.36(4)	7(4)		
					1 S	2.61(4)	8(4)		
Ni(II)	8344.3	5(2)	Yes	5	1 Im 0°	2.17(4)	1(3)		
					1 Im 0°	2.01(3)	1(2)		
					1 N/O	2.04(2)	1(2)	1(2)	2.5
					1 S	2.29(2)	3(2)		
					1 S	2.56(3)	9(3)		
					1 Im 0°	1.94(3)	2(3)		

XANES Analysis					EXAFS Analysis						
Sample	K-edge energy (eV)	1s → 3d peak area (×10 ² eV)	1s → 4p _z observed	Coord #	Shell	r (Å)	σ ² (×10 ⁻³ Å ²)	E ₀ (eV)	%R		
Zn(II)	9662.9	NA	NA	4/5	1 Im 0°	2.08(3)	1(2)	-2(1)	1.1		
					1 N/O	2.02(3)	6(5)				
					1 S	2.29(1)	3(2)				
					1 Br	2.41(1)	7(1)				
					1 Im	1.99(3)	3(5)				
					1 Im	2.11(4)	3(5)				
					1 S	2.27(2)	3(2)			-3(2)	2.3
					1 Br	2.41(1)	6(1)				
					1 Im	1.97(2)	1(3)				
					1 Im	2.09(3)	0(3)				
E63A-RcnR											
Co(II)	7720.6	5(1)	No	6	2 N/O	2.08(2)	2(2)	-2(1)	2.6		
					1 S	2.50(2)	4(2)				
					1 Br	2.45(2)	1(2)				
					1 Im 5°	2.03(3)	4(3)				
					1 Im 10°	2.16(6)	7(7)				
Co(II) ^b	7720.2	6(1)	No	6	1 N/O	1.95(5)	5(6)	-2(2)	2.6		
					1 N/O	2.11(3)	0(3)				
					1 S/Cl	2.66(5)	8(4)				
					1 S/Cl	2.42(4)	8(4)				
					1 Im 0°	2.00(2)	2(2)				
Ni(II)	8343.5	>0.5	No	6	1 Im 0°	2.15(2)	2(1)	3(1)	3.5		
					2 N/O	2.02(2)	3(2)				
					1 S	2.50(4)	2(4)				
					1 Br	2.44(4)	3(4)				
					1 Im 0°	2.03(3)	2(3)				
					1 Im 10°	2.16(2)	1(2)	3(2)	3.8		
					2 N/O	2.01(3)	3(3)				
					1 S	2.38(5)	9(6)				

XANES Analysis					EXAFS Analysis				
Sample	K-edge energy (eV)	1s → 3d peak area (×10 ² eV)	1s → 4p _z observed	Coord #	Shell	r (Å)	σ ² (×10 ⁻³ Å ²)	E ₀ (eV)	%R
Zn(II)	9662.7	NA	NA	4/5	1 S (Cl ⁻)	2.59(3)	5(3)		
					1 Im 0°	2.01(8)	9(7)		
					1 Im 10°	2.08(5)	3(6)		
					1 S	2.23(2)	9(3)	-3(1)	1.1
					1 Br	2.40(1)	4(1)		
					1 Im 0°	2.05(1)	6(2)		
					1 Im 0°	2.17(2)	2(3)		
					1 Im 0°	1.93(1)	4(1)		
					1 N/O	1.99(3)	6(4)	-4(2)	1.0
					1 S	2.24(3)	14(5)		
					1 Br	2.40(1)	4(0)		
					1 Im 0°	2.09(1)	2(1)		
					1 Im 0°	1.96(2)	1(1)		
					1 S	2.23(2)	9(3)	-5(1)	1.8
Co(II)	7720.6	7(1)	No	6	1 Br	2.40(1)	4(1)		
					1 Im	2.07(1)	3(1)		
					1 Im	1.94(1)	2(1)		
					2 N/O	2.12(3)	9(4)	-3(1)	1.8
					1 S	2.28(2)	9(3)		
					1 S	2.52(3)	5(2)		
					1 Im 0°	2.14(2)	0(2)		
Ni(II)	8342.9	<0.5	Yes	5	1 Im 0°	1.98(1)	1(1)		
					1 N/O	2.04(2)	1(2)	0(2)	3.7
					1 S	2.30(3)	4(3)		
					1 S	2.55(4)	8(4)		
					1 Im 0°	1.94(4)	2(3)		
					1 Im 0°	2.08(4)	2(4)		
					1 S	2.29(3)	6(4)	-1(2)	3.4
Zn(II)	9663.0	NA	NA	4/5	1 S	2.29(3)	6(4)	-1(2)	3.4
					1 Br	2.40(2)	8(3)		

E63C-RenR

Sample	XANES Analysis				EXAFS Analysis			
	K-edge energy (eV)	1s → 3d peak area ($\times 10^2$ eV)	1s → 4p _z observed	Coord #	Shell	r (Å)	σ^2 ($\times 10^{-3}$ Å ²)	E ₀ (eV)
					1 Im 0°	2.17(3)	4(3)	
					1 Im 0°	2.05(2)	7(2)	
					1 Im 0°	1.93(2)	4(2)	
					1 N/O	1.96(3)	1(2)	-1(2)
					1 S	2.29(2)	3(2)	4.4
					1 Br	2.42(2)	9(3)	
					1 Im 0°	2.05(2)	0(1)	

^aThe numbers in parentheses are the estimated uncertainties in the corresponding variables. These uncertainties are calculated by Artemis and reflect the change in the variable that will result in an increase in χ^2 of 1. EXAFS fits in bold type are shown in Figures 2–5.

^bSample prepared in buffer with 20 mM Hepes, 300 mM NaCl, 1mM TCEP and 10% glycerol (pH 7.0).

Table 2Magnetic susceptibility data and calculated values for WT- and E63C-*EcRcnR* Ni(II) complexes.

Parameter	WT- <i>EcRcnR</i>	E63C- <i>EcRcnR</i>
ν	-18.4005 Hz	-1.56944 Hz
m	0.0226 g/mL	0.00942 g/mL
M	10,061 g/mol	10,035 g/mol
χ_M	4.89×10^{-3} cgs/mol	9.97×10^{-4} cgs/mol
χ_L	4.48×10^{-6} cgs/mol	4.48×10^{-6} cgs/mol
χ_M'	4.89×10^{-3} cgs/mol	9.93×10^{-4} cgs/mol
μ_{eff}	3.42 BM	1.54 BM

Table 3Distances, angle and dihedral constraints used to guide the modeling of *EcRcnR* metal binding sites. *

Model	Constrained atoms	Distance (Å)
Co(II)- <i>EcRcnR</i>	Co(II) - Ser2(NH ₂)	2.04
	Co(II) - His3(Nδ)	2.04
	Co(II) - Glu34(Oδ1)	2.04
	Co(II) - Cys35(Sγ)	2.31
	Co(II) - Glu63(Oδ1)	2.04
	Co(II) - His64(Nε)	2.04
	His60(Nε) - Cys35(Sγ)	3.00 ± 0.10
Ni(II)- <i>EcRcnR</i>	Ni(II) - Ser2(NH ₂)	2.14
	Ni(II) - Ser2(O)	2.14
	Ni(II) - Cys35(Sγ)	2.63
	Ni(II) - Glu63(Oδ1)	2.14
	Ni(II) - Glu63(Oδ2)	2.14
	Ni(II) - His64(Nε)	2.05
Zn(II)- <i>EcRcnR</i>	Zn(II) - Cys35(Sγ)	2.30
	Zn(II) - Glu63(Oδ1)	2.10
	Zn(II) - His64(Nε)	2.05
	Zn(II) - His67(Nε)	2.05
	Zn(II) - Br ⁻	2.41
Bonded atoms	Constrained atoms	Angle (degrees)
M-Cys(Sγ)	M-Cys(Sγ)-Cys(Cβ)	109 ± 5
M-Glu(Oδ)	M-Glu(Oδ)-Glu(Cγ)	120 ± 5
M-His(Nδ)	M-His(Nδ)-His(Cγ)	120 ± 10
	M-His(Nδ)-His(Cε)	120 ± 10
M-His(Nε)	M-His(Nε)-His(Cδ)	120 ± 10
	M-His(Nε)-His(Cε)	120 ± 10
Bonded atoms	Constrained atoms	Dihedral (degrees)
M-His(Nδ)	M-His(Nδ)-His(Cε)-His(Nε)	180 ± 10
	M-His(Nδ)-His(Cγ)-His(Cδ)	180 ± 10
M-His(Nε)	M-His(Nε)-His(Cε)-His(Nδ)	180 ± 10
	M-His(Nε)-His(Cδ)-His(Cγ)	180 ± 10

* Distances that do not derive directly from experimental data are in bold.

Table 4Analysis of structure-function relationships in metal-coordination by *EcRcnR*

Residue	Ni		Co		Refs.
	XAS	LacZ ^a	XAS	LacZ	
S2-NH ₂	Yes	Yes (A2*)	Yes	Yes (A2*)	35, 47
H3	No	Yes (C,L,E)	Yes	Yes (C,L,E)	35, 47
E34	maybe	No (A,C,Q)	Yes	No (A,C,Q) work, ³⁵	this
C35	Yes	No (A) Yes (L)	Yes	Yes (A,L)	35
H60	No	No (A,N,L) Partial (C)	No	Yes (A,C,N,L)	35, 48
E63	Yes	Partial (A,C,Q)	Yes	No (A) Partial (Q,C)	this work ³⁵
H64	Yes	Yes (C) Partial (L)	Yes	Yes(L) Partial (C)	35, 48
H67	No	No (C, L)	No	No (C, L)	35, 48

^a letters in parentheses indicate one letter code for mutants of the indicated WT residue.