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Evaluation of the Regulatory Model for Ni²⁺ Sensing by Nur from *Streptomyces coelicolor*

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Abstract

Streptomyces coelicolor is a soil-dwelling bacterium that is medically important due to its ability to produce several antibiotics, and nickel accumulation within this organism has been shown to prevent the production of the antibiotic undecylprodigiosin. The transcriptional repressor important in regulation of nickel uptake is the homodimeric Nur, a member of the Fur family. Nur contains two metal-binding sites per monomer: the M-site and the Ni-site. The work described here seeks to determine the roles of each of the metal-binding sites to establish a model of Nur activity through mutational studies, metal titrations, and fluorescence anisotropy. Through these studies, a model of Nur activity is proposed in which femtomolar metal binding to one M-site of Nur prompts DNA-binding, and metal binding to the second M-site fully activates the protein. Evidence is provided that shows cooperative metal binding to the Ni-site, but this process dampens affinity for promoter DNA.

Graphical Abstract

The nickel uptake regulator (Nur) from *S. coelicolor* contains two metal binding sites, the M-site and the Ni-site. This work establishes the Ni-site plays the main sensory role while the M-site is essential for structural integrity of the protein

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1. Introduction

Nickel is beneficial for *Streptomyces coelicolor* in that it may be utilized as a cofactor by a variety of enzymes [1]. On the other hand, excess nickel becomes harmful as a result of nickel binding to the active site of a non-metalloenzyme or the displacement of the essential metal of a metalloenzyme by nickel, which can lead to the formation of reactive oxygen species [2,3]. Furthermore, nickel accumulation within *S. coelicolor* has been shown to limit the production of antibiotics actinorhodin and undecylprodigiosin [4,5]. Due to the dual nature of nickel as both essential and toxic to the cell, it is highly important for bacteria to regulate intracellular levels of nickel.

To allow the organism to thrive in nickel-abundant conditions, a sensitive system has evolved for maintaining nickel ion homeostasis and responding to oxidative stress in *S. coelicolor*. The nickel-uptake regulator (Nur) is a unique member of the Fur family of metalloregulators that, to date, is found exclusively in *S. coelicolor* and is the only Ni^{2+} -sensing member of this family [6]. *In vivo*, the presence of elevated concentrations of cytosolic nickel is required for Nur to prevent transcription. Nickel uptake systems *nikABCDE* and *nikMNOQ* are repressed by Nur, as is the Fe-dependent superoxide dismutase (SOD) encoded by *sodF* [6,7]. Nur also activates *sodN*, the gene that encodes the Ni-dependent SOD, which is translationally regulated via a small regulatory mRNA from the *sodF* transcript [8]. Collectively, these facts indicate that Nur plays a pivotal role in regulating nickel uptake and utilization within *S. coelicolor*.

The crystal structure of the protein, as solved by An et al. [7,9] is shown in Figure 1. X-ray crystallographic and mutational studies have shown that nickel binds to the Ni-site and that the specific amino acids involved in coordinating metal at this site are His70, His72, and His126, along with three solvent ligands, in an octahedral geometry. The crystal structure

shows a zinc ion bound at the M-site. The key amino acids at the M-site are His33, His86, His88, and His90; these coordinate the M-site metal in a square-planar geometry. Additionally, Glu101 is positioned to make an electrostatic interaction with the metal at the M-site and serves as a cap to this site. Although the structure shows Zn^{2+} in this site, the authors argue that square planar coordination geometry makes it likely for Ni^{2+} to bind in this site as well. Unlike the Ni-site, the M-site is highly conserved among most Fur family members and has been determined to be the main regulatory site for many of these proteins [10–12].

The *S. coelicolor* genome contains genes encoding other members of the Fur family, including the zinc-uptake regulator (Zur). Similar to the role of Nur in nickel regulation, dimeric Zur represses a high-affinity zinc-uptake system, among other zinc-dependent enzymes [13]. Mutational studies have uncovered the roles of the metal-binding sites of *ScZur* [14,15]. The C-site is a structural metal-binding site key to dimerization. The M-site is involved in DNA-binding, with metal being present at one M-site of the dimeric Zur allowing for repression of some promoters and metal being present at both M-sites allowing for repression of all Zur-regulated promoters. This type of differential regulation is common among Fur family members [12], and it may be for Nur as well, as expanded upon in this study.

Here, the metal-requirements of Nur to bind the *sodF* promoter *in vitro* provide experimental evidence for a regulatory model that deviates from the literature. The model proposed here describes a system in which the DNA-binding domain (DBD) of Nur is able to interact specifically with DNA upon metal binding to one M-site, which may rigidify the flexible region highlighted in Figure 1. We propose that metal binding to the second M-site of the dimeric protein then increases DNA affinity through rigidification of the DBD of the second monomer. In this study, it is shown that metal bound to the Ni-sites is not required to induce DNA-binding.

2. Experimental Procedures

2.1 Cloning of mutant *nur* gene

The *nur* gene was amplified by PCR from cosmid DNA purchased from Genome Enterprise Limited. The gene was cloned into a pET3a (pET15a for M and Ni mutants) expression vector using the NdeI and BamHI endonucleases and stored in *E. coli* DH5 α cells. Site-directed substitutions of Nur residues were created by a primer extension PCR strategy using forward and reverse primers containing the mutation and flanking T7 promoter and terminator primers. All PCR primers were purchased from Eurofins, and the reactions were carried out using OneTaq 2x Master Mix from New England Biolabs. Mutations generated include the following: H33A, H88A, H90A, H70A, H72A, E101A, H126A, single-site mutants; H70/72/126A triple mutant (referred to as Ni throughout the text); and H33/80/88/90A quadruple mutant (referred to as M throughout the text). A TEV protease site was engineered into the M and Ni mutants between the N-term His-tag and Nur. The sequence of *nur* and each mutant was confirmed by sequencing (Eurofins Genomics).

2.2 Protein purification

E. coli NiCo21 (DE3) cells transformed with a pET3a (pET15a for M- and Ni-*nur*) plasmid containing the *nur* gene or the mutant *nur* gene were grown in Luria-Bertani broth to an OD₆₀₀ of 0.6 and induced with 400 μ M isopropyl β -D-1-thiogalactopyranoside. After incubating for four hours, the cells were harvested by centrifugation. The pellet was collected and resuspended in Buffer A, consisting of 25 mM phosphate, 100 mM NaCl, 2 mM sodium dithionite at pH 8 degassed for 1 hour and the resuspension was supplemented with 1% phenylmethylsulfonyl fluoride and 1% DNase; then, the cells were lysed by sonication and centrifuged. The supernatant was loaded into a Ni²⁺-charged metal-chelate affinity chromatography column equilibrated with Buffer A. A step gradient was used that consisted of a 30 mM imidazole wash followed by elution of Nur with 0.5 M imidazole. Fractions containing Nur were identified by 12.5% SDS-PAGE and further purified using a Superdex G75 gel filtration chromatography column using Buffer A. Fractions containing Nur were again identified by SDS-PAGE and concentrated using a Spin-X UF 10k MWCO Concentrator (Corning). Protein was dialyzed 3x against 300 mL of deoxygenated Buffer B (25 mM Tris, 100 mM NaCl buffer at pH 8) under strict anaerobic conditions. The concentration of monomeric Nur ($\epsilon_{280} = 10,010 \text{ M}^{-1} \text{ cm}^{-1}$ [16]) was determined using a Thermo Scientific NanoDrop 2000c Spectrophotometer. The M and Ni mutants did not bind tightly to the metal affinity column without an engineered His-tag. Consequently, these proteins had an extra step that involved an on-column proteolysis step with TEV protease to remove the purification handle. The identity of the protein was further confirmed by MALDI-TOF mass spectrometry. The purified Nur protein was analyzed by DTNB assay to ensure that oxidation of free thiols was minimal.

2.3 Metal content

To determine the amount of metal bound to Nur upon purification, standard solutions of 0.25, 0.5, 0.75 and 1.0 ppm zinc and of 1.0, 2.0, 3.0, and 4.0 ppm nickel were prepared from AA standards (Alfa Aesar) in 5% HNO₃. The absorbance of each metal present in Nur was measured using a Perkin Elmer 560 flame emission atomic absorbance spectrometer or an Agilent Technologies 700 series ICP OES, per the manufacturers' guidelines. The absorbance of zinc was measured at a wavelength of 202.55 nm, and the absorbance of nickel was measured at a wavelength of 216.555 nm. A standard curve was generated for each sample and metal content of the protein samples were calculated from the standard curve and compared to the known protein concentration.

2.4 Metal binding assays

Ni²⁺ and Zn²⁺ binding constants were determined using a competition assay with the common metal-binding chromophore Mag-Fura-2 (MF2) purchased from Thermo Scientific. Stock solutions of the dye were prepared by dissolving the lyophilized powder in 1 mL of degassed ultrapure water. The concentration of the resulting solution was determined by absorption spectroscopy using an extinction coefficients of 22,000 M⁻¹ cm⁻¹ at 369 nm, provided by the vendor product information [17]. The stock MF2 solution was diluted to 10 μ M in Buffer A. To determine the condition-dependent metal affinities, Ni²⁺ or Zn²⁺ were titrated directly into the solution while monitoring the fluorescence emission at 505 nm over

an excitation range of 250 nm – 450 nm on a PTI spectrofluorometer. Competition assays were carried out in degassed Buffer B under anaerobic condition with 10 μ M MF2 and 10 μ M Nur dimer. The spectral changes were monitored by absorbance spectroscopy at 370 and 320 nm using a Thermo Scientific NanoDrop spectrophotometer. The signal vs. metal concentration was plotted and association constants between Nur and each ion were determined using DynaFit [18,19].

2.5 Metal exchangeability assay by gel filtration chromatography

To examine the lability of metal from Nur, 20 μ M monomeric Nur and 100 mM EDTA were incubated for 16–24 hours with or without *sodF* promoter DNA under anaerobic conditions. These samples were passed through a Sephadex G75 gel filtration column that had been equilibrated with degassed buffer B. 0.5 mL fractions were collected, and the Zn^{2+} content was measured using atomic absorbance spectroscopy.

2.6 Nur titration assay by fluorescence anisotropy

A fluorescein-labeled 32-base pair oligonucleotide of the *sodF* promoter (Operon) was diluted to 12 nM in deoxygenated 25 mM Tris, 100 mM NaCl buffer at pH 8 in a gas-tight quartz cuvette. A 77.0 μ M solution of Nur was titrated into the sample DNA for a total of approximately 7 μ M Nur. The sample was analyzed after each injection using a PTI spectrofluorometer outfitted with excitation and emission polarizers and measured at an excitation wavelength of 495 nm and an emission wavelength of 515 nm. The average anisotropy was calculated for each injection from measurements taken over a sixty-second interval. Fluorescence anisotropy was measured for WT-Nur, both of the previously listed mutants, and WT-Nur in the presence of 20 μ M NiCl_2 . The data collected were fit using DynaFit software.¹⁴ The fitting scheme involved a dimerization equilibrium followed by Nur dimer binding to the DNA.

Data was fit either by fixing K_1 ($K_{\text{dimerization}}$) = 1 μ M and varying the 2nd equilibrium constant or by fixing K_2 ($K_{\text{d}, \text{sodF}}$) = 0.57 μ M and varying the dimerization equilibrium constant. The complete scripts are presented in the supporting materials.

3. Results

Wild type Nur was readily purified by exploiting the natural tendency of the protein to bind metals. A two-step FPLC purification strategy utilizing Ni^{2+} affinity chromatography as a bulk purification step followed by gel filtration chromatography yielded the protein estimated at >95% purity, with the mass confirmed by MALDI-TOF.

3.1 Metal Binding

Each Nur dimer binds up to four metal ions; two in the “M-sites” and two in the “Ni-sites.” Given that Nur readily binds to an immobilized Ni^{2+} ion during purification, at least one of

these four sites is unoccupied as isolated from *E. coli*. Table 1 presents the total metal content of the protein upon purification presented as a ratio of metal per Nur monomer. The data indicate that the wild type protein purifies with a mixture of Zn^{2+} and Ni^{2+} ions and that the total metal content is approximately one metal per monomer (0.3 Ni^{2+} and 0.65 Zn^{2+}) or two metal ions per dimer, in agreement with previous reports [7].

Next, we explored the tendency of Nur to bind additional metals. To determine the affinity of these interactions, a competition assay with MagFura-2 (MF2) was used. This approach relies on accurate binding constants between MF2 and Ni^{2+} under experimental conditions that are identical to those used in this study. To ensure accuracy, these values were determined using the method described by Golynskiy et. al. [20], and K_d values of 1.06 μM and 74 nM were determined for Ni^{2+} and Zn^{2+} , respectively, in excellent agreement with the literature when competition from the Tris buffer is accounted for [20–23]. Figure 2 shows a representative titration of 1 mM Ni^{2+} into 10 μM MF2 and 10 μM Nur dimer. Panel A represents the raw data from the experiment with the expected metal-induced decrease in absorbance at 380 nm and increase around 310 nm. Panel B shows the two signals plotted vs. $[\text{Ni}^{2+}]$ with the lines representing the simulated fit according to a competition model between MF2 and 2 binding sites per Nur dimer. Table 2 summarizes the average best fit of three independent titrations for the first ($K_{d1} = 40$ nM) and second ($K_{d2} = 450$ nM) binding events between metal and Nur. These values represent the buffer-independent binding constants at pH 8.0.

It has been reported that Zn^{2+} is not able to replace Ni^{2+} in the Ni-site of Nur crystals during x-ray crystallography experiments. This is surprising considering the trends in metal affinity predicted by the Irving-Williams series, which indicate that these two metals commonly have similar affinities when interacting with identical sites [24,25]. However, sensory sites in other metalloregulators have evolved to have exquisite selectivity for their target metal [12]. Therefore, the Zn^{2+} affinity of the wild type protein was measured. The first Zn^{2+} binds with a dissociation constant, K_{d1} , or 6.9 nM, roughly 6 times tighter than Ni^{2+} , while the second binds with an affinity that is comparable to the second Ni^{2+} binding ($K_{d2} = 800$ nM).

3.2 DNA Binding

Nur has been shown to directly regulate at least four operons in *S. coelicolor* [7]. To be consistent with previous studies, the *sodF* promoter was selected for DNA-binding experiments [7]. Fluorescence anisotropy was used to monitor complex formation as Nur is titrated into a solution containing fluorescein-labelled dsDNA corresponding to the central 32 nucleotides of the *sodF* promoter, which is sufficient for all molecular contacts to be made based on modelling the protein-DNA interaction. The data for WT-Nur is shown in Figure 3 (black) and is plotted as the fractional saturation of *sodF* dsDNA. The solid line represents a simulated fit to the data according to a model that includes a single Nur dimer binding to the promoter; the average best fit for this interaction is $(1.8 \pm 0.2) \times 10^6$ and the average $K_{d,sodF}$ calculated from 5 independent titrations is $0.57 \pm 0.05 \mu\text{M}$ (Table 3). This interaction occurs in the absence of added metal, demonstrating that WT-Nur is able to bind to the *sodF* promoter with only one metal bound per monomer (two per dimer).

This assay was used to test whether additional Ni^{2+} binding to the Nur dimer impacts its ability to interact with DNA. This was tested by carrying out an identical DNA binding assay that had $10\ \mu\text{M}$ Ni^{2+} added to the protein and DNA solutions. This concentration is sufficient to ensure that both of the additional metal binding sites are saturated based on the metal binding data presented above. The resulting isotherm is presented in Figure 3 (green line). The poor fit (compared to wild type) may result from the increased complexity of the overall equilibria that occur when Ni^{2+} binds to the other sites; the fitting model does not account for these equilibria. Nonetheless, Ni^{2+} present in the two extra metal sites of Nur consistently dampens than the affinity relative to the form of Nur that is overexpressed and purified from *E. coli*. Thus, a Nur dimer with all four metal binding sites occupied destabilizes the protein DNA complex relative to the protein with two metals bound.

3.3 M-site vs. Ni-site

The proposed functional model of Nur posits that the M-site is a structural site that is required for proper protein folding and the Ni-site plays the primary regulatory role. Based on this, Nur presumably purifies with the one metal per monomer populated in the M-site and the additional Ni^{2+} binding equilibria that are observed correspond to populating the Ni-site. However, this model is inconsistent with the DNA binding data presented above. Two alternative models are possible: 1. Nur purifies with metal in the Ni-site instead of the M-site or 2. the Nur dimer is purified with metal populated in a mixture of sites. To test these scenarios, a series of His $\rightarrow$ Ala site directed mutants were investigated that should impact metal binding at one site. The metal occupancy were tested for these mutants and presented in Table 1. The data indicates a significantly lower metalation state for mutants corresponding to the M-site while those of the Ni-site retain most of their metal content. These results suggest but do not confirm that the M-site is likely the primary location of metal upon purification. To test this further, mutants were designed that completely abolish metal binding potential at each site. This was accomplished by mutating all M-site or Ni-site residues to alanine (M or Ni, respectively). This data indicates that the Ni mutant retains nearly all of the metal (0.94 metal:monomer) while the M mutant purifies with only a fraction of the wild type (0.15 metal:monomer).

The DNA binding potential of these mutants was also determined; the average K_d values are summarized in Table 4 and the binding isotherms are available in Figure 3 (M and Ni) or in the supporting information. As expected, each single site mutation has a small dampening effect (2- to 4-fold decrease) on the affinity; however, no individual mutation completely abolishes DNA binding. The M and Ni mutations shown in red and blue in Figure 3 do have a profound impact on DNA binding (10 and $28\ \mu\text{M}$, respectively). Both of these deletion mutants have affinities at or below the level of Nur binding to a randomized piece of DNA of equivalent length.

3.4 Exchangeability of metal in the M-site

The results described above suggest that the Ni-site is not populated with metal upon purification nor is it the regulatory site in Nur; consequently, the M-site must be responsible for sensing Ni^{2+} concentrations. Attempts to completely strip the protein of metal by incubating it in excess EDTA (~ 10 -fold) failed to produce apo-Nur. These experiments

consistently stripped some of the metal, but we were never able to produce a protein with less than 0.5 metal per monomer. Consequently, the affinity of apo-Nur for the *sodF* promoter has not been measured. However, to be an effective regulatory protein, Nur must be sensitive to mass action so that it can release Ni^{2+} from the M-site when cytosolic concentrations fall below threshold levels. Therefore, it is necessary for metals in the M-site to be labile in the protein-DNA complex. This hypothesis was tested by incubating Nur with a 10-fold excess of EDTA with or without the *sodF* promoter followed by gel filtration chromatography to separate Nur from any Zn^{2+} that had been chelated by EDTA. Finally, the metal concentrations of the resulting fractions were tested. Zn^{2+} rather than Ni^{2+} was monitored due to the greater abundance of Zn^{2+} in the as-purified protein, making the Zn^{2+} content a more sensitive handle for total metal content of a sample. The top panel of Figure 4 shows that, in the absence of *sodF*, some Zn^{2+} is released from Nur; however there is still a large amount that remains bound. This result is consistent with the EDTA chelation experiment described above. The bottom panel of Figure 4 shows significantly more metal is released when *sodF* is present in the experiment, as evidenced by the large presence of Zn^{2+} in late-eluting fractions. When DNA is present, the elution profile is nearly an exact sum of Nur (black line) and the *sodF*DNA (blue line), suggesting that the Nur-*sodF* complex has dissociated into Nur and *sodF* after the 24 hour incubation; this is expected if the metal has been removed from the M-site. Essentially all remaining protein-bound Zn^{2+} corresponds to the fractions where only Nur is present. These data quantitatively demonstrate that the presence of *sodF* promoter DNA increases the amount of Zn^{2+} that is released from the protein.

3.5 Dissociation and reformation of the Nur-*sodF* complex

Next, we designed an experiment to directly monitor the stability of the protein-DNA complex when metal is chelated from the M-site. 5 μM Nur was incubated with 10 nM *sodF* which, based on the DNA binding experiments described above, is sufficient to saturate the DNA with Nur and ensure a preformed Nur-*sodF* complex. 1 mM EDTA was added to this solution and the anisotropy was monitored as a function of time. Panel A of Figure 5 shows that, in the absence of EDTA, the complex is stable over the timeframe of the experiment. When EDTA is present, a very slow decrease in anisotropy is observed, consistent with a slow dissociation of the Nur-*sodF* complex. The slow rate of dissociation can be explained when considering that the concentration of the *sodF* promoter is only 10 nM meaning that only a very small fraction of Nur can be stripped at a time making the reaction take hours to complete.

Panel B of Figure 5 shows that the Nur-*sodF* complex is readily reformed when Ni^{2+} is added to a solution containing 10 nM *sodF* and 5 μM Nur that has been treated with 150 μM EDTA (36 hours). Ni^{2+} addition up to 150 μM results in a slow increase in anisotropy followed by a sharp inflection and subsequent saturation. This profile indicates that apo-Nur has a weaker affinity for Ni^{2+} than EDTA, but it is able to compete. During the slow rise (up to 150 μM) most of the metal binds to EDTA and the fraction that binds to Nur induces an increase in anisotropy consistent with a protein-DNA complex forming. The rapid rise at 150 μM occurs when the EDTA is saturated and all additional Ni^{2+} added binds tightly to Nur. This data readily fits to a model that incorporates the formation of a Ni^{2+} -EDTA

complex as well as the requirement for Ni^{2+} binding to Nur prior to formation of the complex with DNA (see supplementary material). The red line shows the best fit to this model where $K_{\text{Ni-Nur}} = (4.7 \pm 0.7) \times 10^{14}$ ($K_d = 2$ fM). In this model, proton competition of EDTA was calculated using the approach described previously [22,23] and binding constants for EDTA from the literature ($\text{pK}_{a1} = 9.52$, $\text{pK}_{a2} = 6.13$, $\log K_{\text{Ni-EDTA}} = 18.4$ [26]).

3.6 The role of the Ni-site residues

The DNA binding assays described above present a counter-intuitive observation that Ni-site residues are essential for DNA binding yet Ni^{2+} binding to this site causes a decrease in the affinity for the *sodF* promoter. To gain further insight into this unexpected result, Nur was docked onto a model of the *sodF* promoter using the NPDock server [27]. The resulting structure, shown in Figure 6A, clearly shows the interactions of the recognition helix with the major groove of DNA. It is also clear that the Ni-site is not shielded by, or in contact with, DNA in this model. Further consideration of the Ni-site residues illuminated an intriguing putative hydrogen-bonding network that is formed in the absence of Ni^{2+} between His70, His72, and His126 of the Ni-site and Glu63 of the recognition helix (Figure 6A inset). We hypothesized that the hydrogen bonds formed by these residues are required to establish the proper orientation for the recognition helix to interact with DNA and that nickel binding to the Ni-site would destabilize the hydrogen bond between His70 and Glu63. Panel B shows the resulting binding isotherm of the E63A mutant in comparison to the wild type and wild type supplemented with 10 mM Ni^{2+} . Notably, the E63A mutant isotherm resembles the profile seen when Ni^{2+} is added to the wild type protein, and the E63A mutant is insensitive to Ni^{2+} addition.

Similar to the Nur binding in the presence of Ni^{2+} (Figure 3, green trace), a poor fit was observed when this data was modelled with a 1 μM dimerization constant for Nur. After attempting multiple fitting models that involved much more complex equilibria, of which none provided a reasonable fit to the data, we tested a model where the dimerization constant is varied and the affinity of Nur for *sodF* is fixed. This model achieved a better fit to the data as represented by the solid lines in Figure 6B with the dimerization constants summarized in Table 5. This model of altered dimerization is only one possible explanation for the profile. Most notable, however, is that the affinity of the E63A mutant mirrors the affinity measured for wild type Nur when exogenous Ni^{2+} is present. Also, the affinity of E63A is not sensitive to Ni^{2+} , thus the unfavorable metal-induced effect on DNA affinity has been lost. Together, this result suggests an equivalence between E63A and fully Ni-loaded WT-Nur, which supports the hypothesis that Ni^{2+} binding interferes with the H-bond network of the Ni-site residues.

4. Discussion

Nur is the only known member of the Fur family that senses Ni^{+2} [6]. The regulatory model proposed by Roe and colleagues posits that the Ni-site is the main metal sensory site of Nur and that the M-site plays a structural role. The crystal structure shows that the Ni-site is in close proximity to the DNA-binding domain (DBD), consistent with its proposed regulatory role [7]. The data presented in this paper provides evidence for an alternate model of Nur

function based on metal affinities and exchangeability, the influence of Ni^{2+} on DNA binding, and site-directed mutations that further explore the conclusions drawn from functional assays.

4.1 The M-site of Nur is a high affinity site that contains metal upon purification and restricts rapid metal exchange with the solvent

As purified, Nur contains one stoichiometric equivalent of metal ion per monomer (~35% Ni^{2+} and ~60% Zn^{2+}). This metal remains after incubation with 500 mM imidazole during and after elution in the metal affinity chromatography purification step. Incubating the protein with 10-fold excess EDTA during or after purification only partially removes the metal from this site (~50%). Single site mutations directed at the M-site and the Ni-site reveal the modifications to the latter does not affect the metalation state of the protein; however, mutations to the M-site result in loss of >70% of the metal upon purification (Table 1). The Ni mutant, which converts all metal coordinating residues in the Ni-site to alanine with no concomitant modification to the M-site, retains stoichiometric metal content upon purification. Conversely, mutation of all four metal binding residues in the M-site (M) abolishes nearly all detectable metal when the protein is purified. These results strongly suggest that Nur is purified with metal bound in the M-site and subsequent metal addition would then populate the Ni-sites of the dimer.

Careful examination of the previously-solved crystal structure (PDB ID: 3EYY) offers support for this conclusion. Figure 7 shows a magnified view of each binding site. The M-site contributes four coordinating residues (His33, His86, His88, His90), and Glu101 offers an additional electrostatic contribution to the binding site and seems to completely shield the metal from solvent. The Ni-site is composed of three coordinating residues (His70, His72, and His126) and is highly solvent-exposed, as indicated by the final three coordination sites of the octahedron being occupied by solvent. Based on this, there is likely an enthalpic preference (more bonds) for metal binding to the M-site relative to the Ni-site. Further, the entropy would also seem to favor the M-site because the metal will be completely desolvated when the M-site is loaded compared to partial desolvation for the Ni-site. In summary, there is likely an entropic and enthalpic preference for metal binding at the M-site and possible kinetic interference to solvent exchange from Glu101. Taken together, this supports the conclusion that, upon purification, the one metal bound per monomer is located at the M-site and that the highly solvent-exposed Ni-site is void of metal after purification.

4.2 Ni^{2+} binds with high affinity to the M-site

In this manuscript, we provide binding affinities for three of the four metal sites in the Nur dimer. Using a competition assay with MagFura-2, dissociation constants of 40 nM and 450 nM were measured (Figure 2 and Table 2). These values are in good agreement with those reported previously using ITC [28]. The authors of that paper attributed the higher affinity interaction to the Ni-site and the lower affinity interaction to the M-site. Using an alternative purification approach, they produced apo-Nur and did a direct titration of Ni^{2+} into the apo protein. Unfortunately, we were not able to replicate this purification strategy; each attempt produced Nur with ~0.5 or ~1 metal bound per monomer. However, the similarity observed

between these alternate techniques affords confidence in the values, despite the inconsistency in stoichiometry. Based on the evidence discussed above indicating that the M-site is occupied with metal upon purification, we attribute these values to cooperative binding to each of the Ni-sites in the dimer.

Using an indirect method that involved titrating Ni^{2+} into a Nur-DNA complex that had been destabilized by incubation with excess EDTA (Figure 5), an affinity of 2 femtomolar was measured for one metal binding in the M-site, likely the highest affinity interaction. Unfortunately, since our data does not rule out that Nur can bind DNA with only one metal per dimer, this experiment could not measure the K_d for the 2nd M-site with confidence. Femtomolar affinity has been observed for the sensory metal in other bacterial metal sensors, including several members of the Fur family [14,15,29,30]. This observation, coupled with the observation that metal binding to the Ni-site results in decreased affinity for DNA, leads us to conclude that the M-site is the regulatory site of Nur (discussed further below).

4.3 The M-site is the regulatory site for Nur

The proposal that the Ni-site is the sensory site for Nur is a very attractive model. This is a unique site in the Fur family and has been clearly demonstrated to bind Ni^{2+} . Further, previous studies provide evidence that deletion of Ni-site residues results in abolished Ni-induced binding to the *sodF* promoter [7]. Taken together, the conclusion that this is the sensory site is logical. However, in this paper, we have provided quantitative evidence that Ni^{2+} is not necessary in the Ni-site for Nur to bind to the *sodF* promoter and that addition of Ni^{2+} has a negative impact on Nur-*sodF* interaction. This discrepancy could be an artifact of the different sizes of DNA used. Ahn *et al.* used a 90 nucleotide fragment corresponding to the -60 to +30 nucleotides relative to the *sodF* start site [7] while we used synthetic 32-nucleotide fragments designed around the core recognition palindrome of the promoter.

The data presented in this manuscript provide evidence to support the conclusion that the M-site is the main regulatory site. As purified, WT-Nur has metal-loaded M-sites and empty Ni-sites and it is able to bind to the *sodF* 32mer with maximum affinity. Additional Ni^{2+} added to protein binds at the Ni-site and decreases the affinity for *sodF* DNA. Thus, the M-site is likely to be the regulatory metal-binding site of Nur. The Nur-*sodF* interaction is completely abolished by adding EDTA to the preformed complex (Figure 4), and addition of Ni^{2+} to a solution that contains presumably apo Nur, EDTA, and DNA triggers the reformation of the Nur-*sodF* complex. In this experiment, apo-Nur must first bind Ni^{2+} with femtomolar affinity prior to the interaction with DNA. This model is consistent with the observation that apo-Fur can only be produced under modestly denaturing conditions and, once obtained, apo-Fur readily rebinds the regulatory zinc ion [35].

5. Concluding remarks

In conclusion, this study supports a model in which Nur adopts a more traditional regulatory strategy of Fur proteins involving a two-conformation model where the regulatory/dimerization domain is linked to the DNA-binding domain (DBD) through a flexible linker. We favor the proposed model for Fur proteins in which the presence of metal induces a

structural change in the protein that adjusts the position of the linker and locks the DBD into a conformation that binds DNA (“open” to “closed” transition [10,12]). In Nur, two of the M-site residues are part of the flexible linker domain while a third is located on the DBD. Upon metal binding to the M-site of Nur, the flexible loop and the residues involved in the adjacent β -hairpin structure are stabilized to put Nur into the “closed” DNA-binding conformation. Metal binding to the M-site of a second monomer would increase DNA affinity through stabilization of the 2nd DBD of the dimer. The role of the Ni-site may be in $[\text{Ni}^{2+}]$ -dependent fine-tuning causing selectivity for the different operons that are regulated by Nur in *Streptomyces coelicolor*, a strategy that has been demonstrated for several Zur proteins [14,15]. This may be facilitated through the subtle H-bond link between the Ni-site and the recognition helix represented in Figure 6A.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Mulrooney SB, Hausinger RP, Nickel uptake and utilization by microorganisms., FEMS Microbiol. Rev 27 (2003) 239–261. [PubMed: 12829270]
- [2]. Stohs SJ, Bagchi D, Oxidative mechanisms in the toxicity of metal ions., Free Radic. Biol. Med 18 (1995) 321–336. [PubMed: 7744317]
- [3]. Macomber L, Hausinger RP, Mechanisms of nickel toxicity in microorganisms., Metallomics. 3 (2011) 1153–1162. doi:10.1039/c1mt00063b. [PubMed: 21799955]
- [4]. Abbas AS, Edwards C, Effects of Metals on Streptomyces coelicolor Growth and Actinorhodin Production, Appl. Environ. Microbiol. 56 (1990) 675–680. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC183404/>. [PubMed: 16348141]
- [5]. Kim HM, Ahn B-E, Lee J-H, Roe J-H, Regulation of a nickel-cobalt efflux system and nickel homeostasis in a soil actinobacterium Streptomyces coelicolor, Metallomics. 7 (2015) 702–709. doi:10.1039/C4MT00318G. [PubMed: 25697558]
- [6]. Ahn BE, Cha J, Lee EJ, Han AR, Thompson CJ, Roe JH, Nur, a nickel-responsive regulator of the Fur family, regulates superoxide dismutases and nickel transport in Streptomyces coelicolor, Mol. Microbiol 59 (2006) 1848–1858. doi:10.1111/j.1365-2958.2006.05065.x. [PubMed: 16553888]
- [7]. An YJ, Ahn BE, Han AR, Kim HM, Chung KM, Shin JH, Cho YB, Roe JH, Cha SS, Structural basis for the specialization of Nur, a nickel-specific Fur homolog, in metal sensing and DNA recognition, Nucleic Acids Res. 37 (2009) 3442–3451. doi:10.1093/nar/gkp198. [PubMed: 19336416]
- [8]. Kim HM, Shin JH, Cho YB, Roe JH, Inverse regulation of Fe-And Ni-containing SOD genes by a fur family regulator Nur through small RNA processed from 3' UTR of the sodF mRNA, Nucleic Acids Res. 42 (2014) 2003–2014. doi:10.1093/nar/gkt1071. [PubMed: 24234448]
- [9]. An YJ, Ahn BE, Roe JH, Cha SS, Crystallization and preliminary X-ray crystallographic analyses of Nur, a nickel-responsive transcription regulator from Streptomyces coelicolor, Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun 64 (2008) 130–132. doi:10.1107/S1744309108001760.

- [10]. Lucarelli D, Russo S, Garman E, Milano A, Meyer-Klaucke W, Pohl E, Crystal structure and function of the zinc uptake regulator FurB from *Mycobacterium tuberculosis*, *J. Biol. Chem* 282 (2007) 9914–9922. doi:10.1074/jbc.M609974200. [PubMed: 17213192]
- [11]. Outten CE, Tobin DA, Penner-Hahn JE, V O'Halloran T, Characterization of the Metal Receptor Sites in *Escherichia coli* Zur, an Ultrasensitive Zinc(II) Metalloregulatory Protein, *Biochemistry*. 40 (2001) 10417–10423. doi:10.1021/bi0155448. [PubMed: 11523983]
- [12]. Ma Z, Jacobsen FE, Giedroc DP, Coordination chemistry of bacterial metal transport, *Chem. Rev.* (Washington, D. C.). 109 (2009) 4644–4681. doi:10.1021/cr900077w.
- [13]. Shin J-H, Oh S-Y, Kim S-J, Roe J-H, The Zinc-Responsive Regulator Zur Controls a Zinc Uptake System and Some Ribosomal Proteins in *Streptomyces coelicolor* A3(2), *J. Bacteriol* 189 (2007) 4070–4077. doi:10.1128/JB.01851-06. [PubMed: 17416659]
- [14]. Shin J-H, Jung HJ, An YJ, Cho Y-B, Cha S-S, Roe J-H, Graded expression of zinc-responsive genes through two regulatory zinc-binding sites in Zur, *Proc. Natl. Acad. Sci.* 108 (2011) 5045 LP–5050. <http://www.pnas.org/content/108/12/5045.abstract>. [PubMed: 21383173]
- [15]. Ma Z, Gabriel SE, Helmann JD, Sequential binding and sensing of Zn(II) by *Bacillus subtilis* Zur, *Nucleic Acids Res.* 39 (2011) 9130–9138. doi:10.1093/nar/gkr625. [PubMed: 21821657]
- [16]. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A, ExPASy: The proteomics server for in-depth protein knowledge and analysis., *Nucleic Acids Res.* 31 (2003) 3784–3788. [PubMed: 12824418]
- [17]. Fluorescent Magnesium Indicators, *Mol. Probes.* (2005) 1–4. <https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp01290.pdf> (accessed May 10, 2018).
- [18]. Kuzmic P, Program DYNAFIT for the analysis of enzyme kinetic data: Application to HIV protease, *Anal. Biochem* 237 (1996) 260–273. [PubMed: 8660575]
- [19]. Kuzmi P, DynaFit-A Software Package for Enzymology, *Methods Enzymol.* 467 (2009) 247–280. doi:10.1016/S0076-6879(09)67010-5. [PubMed: 19897096]
- [20]. Golynskiy MV, Gunderson WA, Hendrich MP, Cohen SM, Metal binding studies and EPR spectroscopy of the manganese transport regulator MntR, *Biochemistry*. 45 (2006) 15359–15372. doi:10.1021/bi0607406. [PubMed: 17176058]
- [21]. Simons TJB, Measurement of free Zn²⁺ ion concentration with the fluorescent probe mag-fura-2 (furaFura), *J. Biochem. Biophys. Methods* 27 (1993) 25–37. doi:10.1016/0165-022X(93)90065-V. [PubMed: 8409208]
- [22]. Johnson RA, Manley OM, Spuches AM, Grosseohme NE, Dissecting ITC data of metal ions binding to ligands and proteins, *Biochim. Biophys. Acta - Gen. Subj* 1860 (2016) 892–901. doi:10.1016/j.bbagen.2015.08.018.
- [23]. Grosseohme NE, Spuches AM, Wilcox DE, Application of isothermal titration calorimetry in bioinorganic chemistry, *J. Biol. Inorg. Chem* 15 (2010) 1183–1191. doi:10.1007/s00775-010-0693-3. [PubMed: 20725755]
- [24]. Grosseohme NE, Akilesh S, Lou Guerinot M, Wilcox DE, Metal-binding thermodynamics of the histidine-rich sequence from the metal-transport protein IRT1 of *Arabidopsis thaliana*, *Inorg. Chem* 45 (2006) 8500–8508. doi:10.1021/ic0606431. [PubMed: 17029360]
- [25]. Irving H, Williams RJP, Order of Stability of Metal Complexes, *Nature*. 162 (1948) 746–747. doi:10.1038/162746a0.
- [26]. NIST, NIST Database 46 Version 8, (n.d.).
- [27]. Tuszyńska I, Magnus M, Jonak K, Dawson W, Bujnicki JM, NPDock: a web server for protein–nucleic acid docking, *Nucleic Acids Res.* 43 (2015) W425–W430. doi:10.1093/nar/gkv493. [PubMed: 25977296]
- [28]. Musiani F, Zambelli B, Bazzani M, Mazzei L, Ciurli S, Nickel-responsive transcriptional regulators, *Metalomics*. 7 (2015) 1305–1318. doi:10.1039/C5MT00072F. [PubMed: 26099858]
- [29]. Choi S-H, Lee K-L, Shin J-H, Cho Y-B, Cha S-S, Roe J-H, Zinc-dependent regulation of zinc import and export genes by Zur, *Nat. Commun* 8 (2017) 15812. doi:10.1038/ncomms15812. [PubMed: 28598435]
- [30]. Outten C, O'Halloran T, Femtomolar sensitivity of metalloregulatory protein controlling zinc homeostasis, *Science* (80-.). 292 (2001) 2488–2492. doi:10.1126/science.1060331.

- [31]. Traoré DAK, El Ghazouani A, Jacquamet L, Borel F, Ferrer JL, Lascoux D, Ravanat JL, Jaquinod M, Blondin G, Caux-Thang C, Duarte V, Latour JM, Structural and functional characterization of 2-oxo-histidine in oxidized PerR protein, *Nat. Chem. Biol* 5 (2009) 53–59. doi:10.1038/nchembio.133. [PubMed: 19079268]
- [32]. Traore DAK, El Ghazouani A, Ilango S, Dupuy J, Jacquamet L, Ferrer J-L, Caux-Thang C, Duarte V, Latour J-M, Crystal structure of the apo-PerR-Zn protein from *Bacillus subtilis*, *Mol. Microbiol* 61 (2006) 1211–1219. doi:10.1111/j.1365-2958.2006.05313.x. [PubMed: 16925555]
- [33]. Pohl E, Haller JC, Mijovilovich A, Meyer-Klaucke W, Garman E, Vasil ML, Architecture of a protein central to iron homeostasis: Crystal structure and spectroscopic analysis of the ferric uptake regulator, *Mol. Microbiol* 47 (2003) 903–915. doi:10.1046/j.1365-2958.2003.03337.x. [PubMed: 12581348]
- [34]. Guerra AJ, Giedroc DP, Metal site occupancy and allosteric switching in bacterial metal sensor proteins, *Arch. Biochem. Biophys* 519 (2012) 210–222. doi:10.1016/j.abb.2011.11.021. [PubMed: 22178748]
- [35]. Althaus EW, Outten CE, Olson KE, Cao H, O'Halloran TV, The ferric uptake regulation (Fur) repressor is a zinc metalloprotein, *Biochemistry*. 38 (1999) 6559–6569. doi:10.1021/bi982788s. [PubMed: 10350474]

Highlights

- The protein contains one metal per monomer (two per dimer) as purified from *E. coli*.
- Two additional Ni²⁺ binds to the dimer with 40 nM and 450 nM affinity.
- Addition of Ni²⁺ decreases the affinity of the protein for the *sodF* promoter.
- Deleting the M-site, but not the Ni-site, abolishes the metal content when purified.
- Occupation of the Ni-site dampens DNA binding affinity.

Figure 1:

The ribbon structure of dimeric Nur. One Nur monomer is shown in black and the other in white. Metal-coordinating residues of the M- and Ni-sites are shown. The flexible loop proposed to be stabilized by metal binding at the M-site is highlighted in yellow.

Figure 2.

Competition metal binding titration between MagFura2 and Nur. 10 μM each Nur dimer and MF2 were titrated with 1 mM Ni^{2+} in 25 mM Tris 100 mM NaCl pH 8.0. Panel A shows the raw spectra, with the initial spectrum shown in red and the spectrum at the conclusion of the titration shown in blue. Panel B shows the absorbance plotted vs. $[\text{Ni}^{2+}]$ overlaid with the best fit to the data according to a competition model that included two independent sites per Nur dimer and MF2.

Figure 3:
DNA binding of Nur. The saturation of DNA by WT-Nur (black), M-Nur (red), Ni-Nur (blue), and WT-Nur with 10 mM supplementary Ni²⁺ (green) with fluorescein-labeled *sodF* promoter.

Figure 4. Relative Zn^{2+} concentration overlaid on a gel filtration (red) trace of Nur with excess EDTA with (bottom) or without (top) *sodF* promoter DNA. The blue and black lines in the bottom panel represent the elution profile of the *sodF* promoter DNA and the Nur protein, respectively, for comparison.

Figure 5.

A: 10 nM sodF and 5 μM Nur incubated in the presence (black squares) or absence (red circles) of 1 mM EDTA. **B:** Ni^{2+} titrated into Nur-sodF complex that has been incubated with 150 μM EDTA. The red line is a best fit according to the model described in the text where $K_{Ni-Nur} = 4.7 \times 10^{14}$ ($K_d = 2.13$ fM). The fractional saturation is calculated from fluorescence anisotropy values determined for each time point.

Figure 6: Nur docked to a model of the *sodF* promoter.

A: The recognition helix of Nur, which interacts with the major groove of DNA and nonspecifically with the backbone, is highlighted in yellow. The inset shows a magnified view of His70, His72, His 126, and Glu63. Potential hydrogen bonds are shown in green [7,27]. **B:** Binding isotherms of wild type (WT) and E63A Nur proteins titrated into the *sodF* promoter in the presence of absence of 10 μM Ni^{2+} .

Figure 7: Magnified views of the metal-binding sites in the crystal structure of Nur.

The backbone of the protein is shown in black, and solvent ligands that coordinate metal at the Ni-site are shown in light grey. Heteroatoms are also colored, with red being oxygen and blue being nitrogen. **A:** The M-site with metal-coordinating residues shown. **B:** The M-site with the surface of the protein shown with 30% transparency, showing that the M-site metal is shielded from solvent. **C:** The Ni-site with metal-coordinating residues shown. Solvent molecules from the crystallization matrix, ethylene glycol and malonate coordinate nickel at the Ni-site. **D:** The Ni-site with the surface of the protein shown with 30% transparency, showing that the Ni-site is exposed to solvent. Solvent molecules ethylene glycol and malonate are shown in grey.

Table 1.

Summary of Metal Occupancy of WT-Nur and Nur mutants. The reported metal occupancy is reported as a ratio of metal to Nur monomer.

	WT	Ni-site Mutants				M-site Mutants				
		H70A	H72A	H126A	Ni	H33A	H86A	H88A	H90A	M
Ni²⁺	0.3	0.1	0.17	0.1	0.23	0	0	0	0	0
Zn²⁺	0.65	0.9	0.79	0.9	0.71	0.3	0.2	0.3	0.2	0.15
Total	0.95	1.0	0.96	1.0	0.94	0.3	0.2	0.3	0.2	0.15

Table 2.

Summary of the affinities for metal of WT-Nur and deletion mutants.

Mutant	K _{d1} (nM)	K _{d2} (nM)	K _{d2} /K _{d1}
	Ni ²⁺ Binding		
WT-Nur	40 ± 2	450 ± 40	12
	Zn ²⁺ Binding		
	6.9 ± 0.5	800 ± 300	115
Ni	No binding detected		
M	2000 ± 2500		N/A

Table 3.

Summary of average dissociation constants for Nur binding to the *sodF* promoter or a randomized double stranded 32mer.

DNA	Protein	$K_{d,sodF}$ (μ M)
<i>sodF</i>	WT	0.57 ± 0.05
	WT + 10 μ M Ni ²⁺	1.1 ± 0.9
<i>Random 32mer</i>		9 ± 1

Table 4.

Summary of average dissociation constants (K_d in μM) for Nur mutants binding to the *sodF* promoter

Ni-site Mutants				M-site Mutants				
H72A	H126A	H33A	Ni	H86A	H88A	H90A	M	E63A
1.2 ± 0.4	0.9 ± 0.1	1.0 ± 0.3	28^a	0.92 ± 0.1	1.1 ± 0.3	0.92 ± 0.1	10 ± 1	1.5 ± 0.4

^aThis value represents a minimum threshold due to very weak binding.

Table 5.

Summary of binding titrations fit with fixed Nur-*sodF* affinity ($K_d = 0.57 \mu\text{M}$) and varying dimerization constants.

Protein	$K_{\text{dimerization}}$
Wild type	1 μM (fixed for reference)
Wild type + 10 μM Ni^{2+}	$9.0 \pm 0.1 \mu\text{M}$
E63A	$15 \pm 2 \mu\text{M}$
E63A + 10 μM Ni^{2+}	$17 \pm 1 \mu\text{M}$