A Nickel-Cobalt-sensing ArsR-SmtB Family Repressor

CONTRIBUTIONS OF CYTOSOL AND EFFECTOR BINDING SITES TO METAL SELECTIVITY

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NmtR from Mycobacterium tuberculosis is a new member of the ArsR-SmtB family of metal sensor transcriptional repressors. NmtR binds to the operator-promoter of a gene encoding a P₁ type ATPase (NmtA), repressing transcription in vivo except in medium supplemented with nickel or, to some extent, cobalt. In a cyanobacterial host, Synechococcus PCC 7942 strain R2-PIM8(smt)

MntR-mediated repression is alleviated by cobalt but not nickel or zinc addition, while the related sensor SmtB responds exclusively to zinc. Quantification of the number of atoms of nickel per cell shows that NmtR nickel sensitivity correlates with cytosolic nickel contents. Differential metal discrimination in a common cytosol by SmtB (zinc) and NmtR (cobalt) is not simply explained by affinities at equilibrium; although NmtR does bind nickel substantially more tightly than SmtB, it has a higher affinity for zinc than for cobalt and binds cobalt more weakly than SmtB. SmtB is known to bind and sense zinc at interhelical four-coordinate, tetrahedral sites across the C-terminal α5 helices, while absorption spectroscopy of Co(II)- and Ni(II)-substituted NmtR reveals five- and six-coordinate metal complexes. Site-directed mutagenesis identifies six potential cobalt/nickel ligands that are obligatory for inducer recognition but not repression by NmtR, four of which (Asp⁹¹, His⁹³, His¹⁰⁴, His¹⁰⁷) align with α5 ligands of SmtB with two additional His provided by a carboxyl-terminal "extension" (designated α5C). Gel retardation assays reveal that zinc does not allosterically regulate NmtR-DNA binding at concentrations where lower affinity cobalt does. These data suggest that two additional ligands form hexacoordinate metal complexes and are crucial for driving allosteric regulation of DNA binding by NmtR, thereby allowing NmtR to preferentially sense metals that favor higher coordination numbers relative to SmtB.

Cells contain regulatory proteins to detect and respond to deficiency or excess of essential metals to maintain sufficient atoms to satisfy the requirements of metalloproteins while avoiding toxicity (1). The ArsR-SmtB family of transcriptional repressors associate with the promoters of genes encoding proteins involved in the efflux and/or sequestration of excess metal (2). De-repression occurs when the repressors bind metal effectors coincident with the number of atoms exceeding an optimal cell quota. SmtB-mediated repression is alleviated by Zn(II) (3), ZiaR by Zn(II) (4), ArsR by As(III), Sb(III), and Bi(III) (5), CadC by Cd(II), Pb(II) and Bi(III) (6–8), and CzrA by Co(II) and Zn(II) (9, 10). Clearly these sensors discriminate between different metals in vivo, but the factors dictating which inorganic elements elicit responses remain to be defined.

There is rich literature describing metal coordination by numerous small molecules in vitro and established theories cataloguing the factors likely to influence metal selectivity in vivo (11). The ligand environments of metal ions are also known in a vast array of metalloenzymes (12). The binding sites of enzymes are not only influenced by metal selectivity but also by catalytic constraints, different secondary and tertiary structures, and evolutionary histories. Metalloregulatory proteins have some advantages for exploring metal selectivity in vivo. First, selectivity will have been a dominant factor in the evolution from a common ancestor of structurally similar sensors that detect different metals. Second, by associating their target promoters with reporter genes it is possible to monitor metal occupancy in vivo. In some, if not all, cells there is an absence of free copper (13), and it is likely that this is also true of several other essential metals including zinc (14). Thus, factors that influence the probability of sensors encountering different labile metal ions are likely to influence metal specificity. For example, metallochaperones assist in the delivery of metals, including nickel (15) to some proteins or target compartments (16), promoting advantageous metal-protein partnerships while inhibiting others en route. In a two-hybrid assay, a copper metallochaperone from Synechocystis PCC 6803 was shown to interact with copper transporting P₁-type ATPases but not with structurally related zinc or cobalt transporters (17), which illustrates how the specificity of metallochaperone-metalloprotein interactions could define which metals are acquired by which proteins in vivo.

To identify the regulatory metal binding sites of SmtB we previously generated mutants of Cys and His candidate ligands. One, or both, of a pair of His residues (105/106) was/were required for metal recognition but not repression (18). Difference electron density maps obtained after soaking apo-SmtB crystals with mercuric acetate suggested two symmetry-related pairs of metal binding sites per dimer (19). A pair of metal sites was located close to the α3 helix within the DNA-binding helix-turn-helix motif and a second pair was formed by four ligands, two from each monomer, bridging antiparallel...
carboxyl-terminal α5 helices. Fractional occupancies were low (<2%), and it was subsequently established that SmtB binds only one zinc per monomer with affinity \( K_{d} \) in excess of \( 10^{11} \) M\(^{-1} \) at equilibrium (20). Substitution of cobalt into the zinc sites of SmtB gave spectral features diagnostic of tetrahedral coordination environments with one or two Cys ligands (20). Zinc and cobalt x-ray absorption spectroscopy (21) coupled with \( ^{119}\)Sn–H NMR perturbation spectroscopy (21) implicate Cys\(^{14} \), His\(^{18} \), and Cys\(^{61} \) in a site designated α3N (Fig. 1A). However, Cys variant proteins, deficient in α3N, retain inducer responsiveness in vitro (18) and zinc-dependent DNA dissociation in vitro (22). In contrast H106Q SmtB was refractory to zinc-induced disassembly of SmtB-DNA complexes in vitro (22) consistent with loss of inducer recognition in vitro (18). Occupancy of α5 sites (which include His\(^{106} \), Fig. 1A) regulates DNA binding by SmtB even though α3N sites are occupied in dissociated SmtB (Fig. 1B). In contrast, trinodal thiolate sites adjacent to a predicted helix-turn-helix region are required for inducer recognition by ArsR (23), while a tetrahedrate α3N site modulates CadC DNA binding in vitro (8, 24) and CadC inducer recognition in vivo (7). Different allosteric sites (α3N or α5) with distinct ligand sets and geometries correlate with, and presumably contribute toward, the biological metal specificities of individual ArsR-SmtB family members. Analogous observations have been made for Escherichia coli Fur homologues, Fur and Zur (25).

To identify ArsR-SmtB sensors with new specificities, sequence databases were searched for smtB-related genes adjacent to genes predicted to contribute to homeostasis of other metals. Ten genes were identified in the fully sequenced genome of Mycobacterium tuberculosis (26), and Rv3744 was selected due to its proximity to a divergently transcribed gene (Fig. 1C) encoding a deduced protein with similarity to CoaT. Ten genes were identified in the fully sequenced genome of Mycobacterium tuberculosis (26), and Rv3744 was selected due to its proximity to a divergently transcribed gene (Fig. 1C) encoding a deduced protein with similarity to CoaT. Ten genes were identified in the fully sequenced genome of Mycobacterium tuberculosis (26), and Rv3744 was selected due to its proximity to a divergently transcribed gene (Fig. 1C) encoding a deduced protein with similarity to CoaT.

Experimental Procedures

**Bacterial Strains and DNA Manipulations—**Mycobacterium smegmatis mc\(^{155} \) and M. bovis BCG (Pasteur) were used as mycobacterial hosts, and Synechococcus PCC 7942 strain R2-PIM8(smt) (29), lacking functional smtA and smtB genes, was used as a cyanobacterial host. The smtB- deficient strain of the latter alleviates any influence of SmtB (with a similar recognition helix to NmtR) on expression from the nmtA operator-promoter in this host. Mycobacterial cells were grown with shaking at 37 °C in LB medium (30) containing 0.05% (v/v) Tween 80, and cyanobacterial cells were grown at 30 °C in Allen’s medium using conditions as described (29). E. coli strains JM109 (Stratagene) and BL21(DE3) were used and grown in LB medium. Cells were transformed to antibiotic resistance as described (29–31). Standard DNA manipulations were performed as described by Sambrook et al. (30). All generated plasmid constructs were checked by sequence analysis.

**Construction of nmt-lacZ Fusions, Site-directed Mutagenesis, and β-Galactosidase Assays—**M. tuberculosis H37Rv genomic DNA was used as template for PCR with primers I (5'-GAAGATCCGGCCGCAA-3') and II (5'-CATATGATGCGGACCG-3') and the amplification product (497 bp) containing the nmtA operator-promoter and nmtR (Fig. 1C) ligated to pGEM-T prior to subcloning into the SalI/BamH1 site of pLACBP2 (32) or the Scal/BamH1 site of pJEM15 (31) to create transcriptional fusions with lacZ. “QuickChange” (Stratagene) site-directed mutagenesis was subsequently used, according to the manufacturer’s protocols, to generate derivatives with codon changes (32) to: Gly\(^{143} \), Asp\(^{153} \), Asp\(^{156} \), and Asp\(^{111} \) to Ala; and His\(^{104} \), His\(^{117} \), His\(^{109} \), and His\(^{115} \) to Arg. The pLACBP2- and pJEM15-based constructs were introduced into cyanobacterial and mycobacterial hosts, respectively. R2-PIM8(smt) containing smtB and the smtA operator-promoter in pLACBP2 (3) was used to examine expression from the smtA operator-promoter. β-Galactosidase assays were performed as described previously (33), in triplicate on at least three separate occasions. The media were supplemented with various [metal] (described in individual experiments) for approximately 20 h immediately prior to assays. These assays therefore differed from previous reported assays (3, 29, 33), to examine expression from the smtA operator-promoter, which used much shorter (2 h) metal exposure times. Maximum permissive [metal] was: 0.25 μM zinc, 1/200 μM cobalt, 5/500 μM copper, 0.15/500 μM nickel, 0.8/0.5 μM silver, 1.5/2.5 μM cadmium, 100/untested μM lead for cyanobacteria/mycobacteria.

**Gal Retardation Assays—**For these experiments recombinant NmtR was generated as a fusion to glutathione S-transferase by subcloning the nmtR coding region, generated by PCR using primers II and HI (5'-GAAGATCCCATGACCGGACCGGTCAGAG-3') with M. tuberculosis H37Rv DNA as template, into the BamHI/EcoRI site of plasmid pGEX-6P2 (Amersham Biosciences). Recombinant fusion protein was expressed in E. coli JM109, cleaved using precision protease, purified according to the manufacturer’s protocols, and dialyzed against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, followed by 10 mM Tris-HCl (pH 7.5). A single prominent band corresponding to the predicted size of NmtR and five residues of glutathione S-transferase (13258-kDa) was detected by PAGE. Crude cyanobacterial cell extracts were prepared as described (33) and protein concentrations determined using Coomassie Blue R-250 (using bovine serum albumin standards). Gel retardation assays were performed (18) with EDTA omitted from buffers unless otherwise stated. The probe in all cases was 71-bp Xbal/BamHI fragment from pJEM-T containing the nmt operator-promoter region generated by Primers I and HI (5'-GAATTCATGACCGGACCGGTCAGAG-3') with M. tuberculosis H37Rv DNA as template. Examination of metal-induced DNA dissociation involved adding increasing [Co(II)] or [Zn(II)] to binding reactions containing recombinant NmtR; to check for reversibility 1 mM EDTA was also added to some reactions after 50 min.

Expression and Purification of NmtR—The nmtR coding region was amplified by PCR from M. bovis BCG genomic DNA, using primers 5'-GAAGATCCCATGACCGGACCGGTCAGAG-3' and 5'-GAAGATCATGACCGGTCAGAG-3', with M. tuberculosis H37Rv DNA as template. Examination of metal-induced DNA dissociation involved adding increasing [Co(II)] or [Zn(II)] to binding reactions containing recombinant NmtR; to check for reversibility 1 mM EDTA was also added to some reactions after 50 min.

Direct Metal Binding of NmtR and SmtB—Titration of apo-NmtR with [metal] (≤0.5 mol eq of Zn(II)) as determined by atomic absorption spectroscopy, \( \varepsilon_{\text{molar}} = 4470 \) M\(^{-1} \) cm\(^{-1} \) with Ni(II) and Co(II) were monitored by UV-visible optical absorption spectroscopy (600 μM NmtR) or by steady-state tyrosine fluorescence (for Ni(II), Co(II), and Zn(II)); 5.0 μM NmtR) in 10 mM HEPES (pH 7.0), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and elution into the same buffer but containing 300 mM NaCl; fractionation of pooled eluate on Sephadex G-75 (246 ml); concentration by re-application to heparin-Sepharose in an anaerobic chamber; and final application and elution from Sephadex G-25 (Amersham Biosciences) in 20 mM MES-\( \Phi \) (pH 6.0), 150 mM NaCl. A single prominent band of the anticipated size (12.842 kDa) was detected by PAGE. 1° The abbreviation used is: MES, 4-morpholinethanesulfonic acid.
pressing from pET29a, prepared as described for NmtR, and preincubated in an anaerobic chamber with a 4-fold molar excess of Co(II). Studies of competitive metal binding to NmtR (Zn(II) versus Co(II)) involved titrating apo-NmtR in 20 mM MES (pH 6.0), 150 mM NaCl, versus Ni(II), and Zn(II), and measuring [metal] by atomic absorption on Sephadex G-25.

A

**FIG. 1.** Metal binding sites of SmtB and physical map of the nmtR and nmtA genes from *M. tuberculosis*. A, residues assigned (21) to α3N, α5’, and α5 sites are indicated on a ribbon representation of the apo-SmtB homodimer (19), with * representing ligands contributed by the second monomer. Glu120 or Cys121 donate the fourth ligand to Co(II) or Zn(II) at α5’ or α5, respectively. p-(Hydroxymercueryl)phenylsulphonate (PMPs) causes occupancy of α5’. B, in the model, apo-SmtB binds the smt operator-promoter inhibiting smtA transcription, Zn(II) binds at α5 (KZn = 7.8(±1.9) × 10\(^{-13}\) M\(^{-1}\)) (21) causing DNA dissociation and transcription prior to migration to α3N (KZn = 1.0(±0.1) × 10\(^{-13}\) M\(^{-1}\)) (22). C, the nmtR and nmtA genes from *M. tuberculosis*, corresponding to open reading frames Rv3744 and ctpd, respectively, in the *M. tuberculosis* genome (26), are shown. NmtR is predicted to bind to nucleotides within a degenerate 12-2-12 inverted repeat (sequence shown in full) within the nmt operator-promoter region that separates nmtR and nmtA.

**RESULTS**

**NmtR binds to the nmt operator-promoter region and is a nickel- and cobalt-responsive repressor.**—Similarity of the deduced product of open reading frame Rv3744 (NmtR) to the zinc-responsive repressor SmtB (3), and of the divergently transcribed gene (*nmtA*) to metal-transporting P\(_{70}\)-type ATPases, suggests that the former might bind to the intervening operator-promoter region to regulate the latter. To test whether or not NmtR binds to the operator-promoter region separating nmtR and nmtA (Fig. 1C), NmtR was expressed in *E. coli*, purified, and used in gel retardation assays. A single retarded complex was detected (Fig. 2A), which was retained in the presence of nonspecific (poly(dI-dC)-poly(dI-dC)), but not specific (nmt operator promoter region), competitor DNA as probe; the position of free probe (FP) is indicated. B and C, β-galactosidase activity in mycobacterial cells containing nmtR. Cells were grown with no metal supplement and maximum permissive [metal] (B) or up to inhibitory [Ni(II)], [Co(II)], or [Zn(II)] (C); maximum and half-maximum permissive [metal] are indicated (H and L, respectively). Insets, OD\(_{595}\) cultures (y axis) against added [metal] (x axis).

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**DISCUSSION**

*NmtA* shows greater similarity to the cobalt exporting *P. Idaho* ATPase CoaT (27) than to related zinc exporters ZntA (34, 35) or ZiaA (4). It is probable that the same metals will be sensed by NmtR and transported by NmtA and therefore speculated that metal specificities of at least one of the proteins differ from those naively inferred from homologies. To establish which (if any) metals induce transcription from the *nmtA* operator-promoter, a 497-bp region including the operator-promoter separating the two genes plus the entire *nmtR* coding region was fused to a promoterless lacZ (20), which was retained in the presence of nonpermissive (poly(dI-dC)-poly(dI-dC)), but not specific (nmt operator promoter region), competitor DNA (data not shown).

Exposure to a range of concentrations of zinc, cobalt, and nickel negatively toward expression from the *nmtA* operator-promoter, a 497-bp region including the *nmtR* coding region was fused to a promoterless lacZ (20), which was retained in the presence of nonpermissive (poly(dI-dC)-poly(dI-dC)), but not specific (nmt operator promoter region), competitor DNA (data not shown).

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The nickel contents of both organisms were determined in cultures grown in normal medium or following supplementation with maximum permissive (labeled H on Figs. 2 and 4) or half-maximum permissive (L) nickel concentrations. Values were expressed as (i) number of atoms per cell (for the cyanobacterium) or per cyanobacterial cell volume equivalent for the mycobacterial cells and (ii) relative to protein content. These values increase by 37- and 38-fold in the mycobacterium but only 3.5- and 3-fold in the cyanobacterium following nickel supplementation. No increase was detected between half-maximum permissive and maximum permissive concentrations in the cyanobacterium. It is inferred that NmtR does not respond to nickel in the cyanobacterium, because the metal is excluded from the cell, relative to the mycobacterium.

**SmtB Outcompetes NmtR for Zinc**—Table I shows no significant increase in the zinc content of zinc-supplemented mycobacterial cells, but shows a greater than 10-fold increase in cyanobacteria. Exclusion of zinc from the mycobacterium could theoretically have accounted for the lack of a response of NmtR to zinc. Only by having established that NmtR also fails to respond to nickel in the cyanobacterium, will it be concluded that this difference in selectivity reflects intrinsic differences between SmtB and NmtR. A simplistic explanation is that SmtB has a high affinity for zinc, low affinity for cobalt, and NmtR has a low affinity for zinc but high affinity for cobalt. The heirarchy of affinities for SmtB is Zn(II) > Co(II) > Ni(II) (20). To establish the heirarchy of metal binding to NmtR, competitive binding experiments and direct titrations (see be-
SmtB can compete with endogenous ligands, implying that while both proteins bind zinc with highest affinity, the difference in affinity of at least one order of magnitude. Zinc binds to SmtB in preference to NmtR and implying a favor binding of nickel over cobalt, (ii) disfavor binding of zinc and indicating the order Zn(II) > Ni(II) > Co(II). It is possible that while both proteins bind zinc with highest affinity only SmtB can compete with endogenous ligands, implying that NmtR binds zinc with lower affinity than SmtB. The two α5 sites of SmtB, which mediate zinc-dependent DNA dissociation, have $K_{Zn} \approx 7.8 \times 10^{13} \text{M}^{-1}$. The α3N sites that are occupied in free solution have $K_{Zn} \approx$ of at least $10^{13} \text{M}^{-1}$ (21). The zinc-binding site(s) of NmtR remain to be defined, and $K_{Zn}$ may also exceed $10^{13} \text{M}^{-1}$; competitive binding was therefore used to directly establish which protein preferentially acquires zinc.

Cobalt-SmtB is blue with distinctive spectral features around 550 nm, and between 300 and 400 nm (20). In contrast, cobalt-NmtR is nearly colorless with molar absorptivities in the visible region of $\approx 100 \text{M}^{-1} \text{cm}^{-1}$ (see below). Addition of zinc displaces cobalt and thereby bleaches cobalt-SmtB (20). Zinc-mediated bleaching of cobalt-SmtB was unaffected by the presence of an equimolar amount of NmtR (Fig. 5), establishing that zinc binds to SmtB in preference to NmtR and implying a difference in affinity of at least one order of magnitude.

At Equivalent Concentrations Cobalt, but Not Zinc, Cause Dissociation of NmtR from DNA—Of the three ions tested, zinc binds to NmtR with the highest affinity, cobalt the lowest, and yet the latter alleviates NmtR-mediated repression in vivo but the former does not (Fig. 4). Perhaps cobalt-NmtR undergoes an allosteric change to impair DNA binding, while zinc-NmtR does not. This was tested in gel retardation assays. Purified NmtR remains associated with nmt operator-promoter DNA in the presence of zinc at concentrations where cobalt inhibits complexes (Fig. 6). Higher zinc concentrations inhibited the formation of NmtR DNA complexes, but this requires further investigation due to evidence of protein precipitation. Preliminary titration of DNA with preformed complexes of apo-, cobalt-, nickel-, or zinc-NmtR, monitored via fluorescence anisotropy, was also consistent with nickel and cobalt similarly regulating complex formation with both metals more effective than zinc (data not shown).

Identification of Six α5C Residues That Are Essential for Nickel and Cobalt Sensing by NmtR—Some difference in the effector recognition site of NmtR compared with SmtB might (i) favor binding of nickel over cobalt, (ii) disfavor binding of zinc in competition with SmtB (Fig. 5B), and/or (iii) favor allosteric regulation by nickel and cobalt in preference to zinc (Fig. 6).

The next challenge was to identify the inducer recognition site of NmtR. Asp^51 in NmtR aligns with conserved Asp residues in other family members (8) and, at least in SmtB, is thought to contribute one α3N ligand (21). Ala substitution of Asp^51 did not impair either NmtR-mediated repression or nickel/cobalt recognition (Fig. 7). Inducer recognition by NmtR either does not involve α3N sites, or Asp^51 is not an essential α3N ligand.

While inducer recognition by many ArsR-SmtB family members requires α3N sites (8, 23), in SmtB ligands from antiparallel α5 helices at the carboxyl-terminal dimer interface are obligatory for metal-mediated DNA dissociation (21). The α5 helices of NmtR were predicted based upon the coordinates for SmtB, and Fig. 7 shows a hypothetical dimer interface at the carboxyl-terminal region of NmtR. Four candidate nickel/cobalt ligands in NmtR, Asp^94, His^103, His^104, and His^107 (Fig. 7) correspond to the α5 residues Asp^94, His^103, His^117, and Glu^120 of SmtB (Fig. 1A). Substitution of any one of these residues in NmtR created functional repressors that mediated low expression of lacZ from the nmtA operator-promoter in mycobacterial cells grown in the absence of metal supplements. In contrast β-galactosidase activity was constitutively elevated in cells containing a non-functional mutant in which the codon for Gln^11 had been substituted with a stop codon. Most importantly, substitution of NmtR residues aligning with the α5 ligands of SmtB caused loss of inducer recognition with β-galactosidase activity remaining low in the presence of nickel and cobalt concentrations that cause loss of repression by wild-type NmtR.
NmtR has 11 additional carboxyl-terminal residues relative to SmtB (drawn as unstructured ribbon on Fig. 7), including three additional potential nickel/cobalt ligands, His109, Asp114, and His116. Substitution of Asp114 had no detectable effect on repression or inducer recognition, but substitution of either His created inducer non-responsive functional repressors, thereby identifying a total of six residues, all of which are obligatory for either cobalt or nickel recognition (Fig. 7).

UV-visible Absorption Spectroscopy of Ni(II)- and Co(II)-substituted NmtR—Site-directed mutagenesis of NmtR (Fig. 7) suggests that inducer recognition could involve hexadentate Ni(II) or Co(II) coordination complexes formed by extended carboxyl-terminal α5C sites in a way in which six ligands (rather than four for SmtB) are required for allostERIC regulation of DNA binding, and thus sensing. Consistent with this, the saturated UV-visible absorption spectrum of Ni(II)-NmtR (Fig. 8A) recorded at a 1:1 Ni(II):NmtR monomer ratio reveals three very weak (ε ≈ 80 M⁻¹ cm⁻¹) and very broad ligand field absorption transitions diagnostic (36) of six-coordinate d⁸ Ni(II). It is noted that the gradual upward slope in the corrected spectrum of Ni(II)-NmtR is not due to light scattering, but rather to the non-resolved nature of low intensity absorption bands characteristic of Ni(II) in coordination complexes deviating from perfect trigonal bipyramidal or octahedral coordinate symmetry (37). The inset (Fig. 8A) reveals that the stoichiometry of Ni(II) binding to NmtR is 1 Ni(II) per monomer or 2 per dimer (NmtR is fully dimeric under these conditions)² with a lower limit of the affinity for Ni(II), Kₙi ≈ 2 × 10⁷ M⁻¹. For SmtB, Kₙi = 1.7 (± 0.3) × 10⁷ M⁻¹ (20). Analogous data are shown for Co(II)-substituted NmtR in Fig. 8B. Although both 1:1 and 2:1 Co(II):NmtR dimer complexes are spectroscopically indistinguishable (in contrast to Ni(II) complexes), the low molar absorptivities of each complex are indicative of five- or six-coordinate d⁷ Co(II) (38); the spectrum that characterizes the second bound Co(II) is nearly superimposable with other octa-

² M. A. Pennella and D. P. Giedroc, unpublished observations.
hedral Co(II) complexes described in the literature (39). Consistent with the competitive metal binding experiments described above, Co(II) binds to NmtR with an affinity $\geq 40$-fold weaker than Ni(II) and at least 500-fold weaker than Zn(II) (inset, Fig. 8B) again implying that for NmtR, $K_{Co} > K_{Ni} > K_{Zn}$. Remarkably, $K_{Co}$ for NmtR is $\sim 3000$-fold smaller for NmtR relative to SmtB under similar solution conditions (20, 21) despite the finding that NmtR senses Co(II), while SmtB does not (Fig. 3).

DISCUSSION

Several lines of evidence demonstrate that NmtR is a nickel/cobalt-responsive DNA-binding repressor of transcription from the divergent nmtA operator-promoter. (i) Purified NmtR forms specific complexes with the nmtA operator promoter in vitro (Fig. 2A); (ii) equivalent complexes are detected using crude lysates of R2-PIM8(smt) containing NmtR but not cells containing an internal stop codon in nmtR (Fig. 3A); (iii) expression of $\beta$-galactosidase activity from the nmtA operator-promoter is elevated in mycobacterial (Fig. 7) and cyanobacterial (Fig. 3) cells devoid of functional nmtR compared with cells containing NmtR; (iv) expression of $\beta$-galactosidase activity via the nmtA operator-promoter is elevated in both bacterial cell types in response to elevated cobalt (Figs. 2 and 3), and nickel is the most potent inducer at viable concentrations in the mycobacterial (Fig. 7) and cyanobacterial (Fig. 3) situations at the level of protein-metal binding. It remains unclear whether the affinity zinc does not associate with NmtR in vivo in such a way that detection of nickel and cobalt is inhibited. This work highlights the need to identify the chemical form(s) of the labile pool(s) of metals accessible by NmtR.

Why is cobalt (and nickel) more effective than zinc at prompting DNA dissociation by NmtR? NmtR absorption spectra are indicative of distorted octahedral Ni(II) coordination and five- or six-coordinate Co(II) liganding (Fig. 8). In contrast, SmtB is characterized by four-coordinate, tetrahedral complexes of both Co(II) and Zn(II) (20, 21). Although the coordination geometry of Zn(II)-NmtR is not yet known, at least one explanation is that the difference in the allosteric regulation of DNA binding by NmtR versus SmtB requires metal coordination bonds to six protein-derived ligands, rather than four. This is consistent with the identification of six potential ligands by site-directed mutagenesis, each obligatory for inducer recognition in vivo. Ni(II) and Co(II) show a greater propensity to form octahedral coordination complexes relative to Zn(II) (11), and this is the most common geometry for Ni(II) and Co(II) cataloged in protein structural databases (12); in contrast, tetrahedral coordination geometry predominates for Zn(II) (12). Consistent with these trends, Zn(II) complexes of $E$. coli glyoxalase are five-coordinate and catalytically inactive, whereas the Ni(II) and Co(II) complexes recruit an additional water molecule into the first coordination sphere creating a nearly perfect octahedral complex, which exhibits high catalytic activity (41). It is tempting to speculate that two additional ligands provided by the COOH-terminal extension of the $\alpha$ helices in NmtR (Fig. 7) adapt negative regulation of operator/promoter binding previously observed for SmtB (22) to require hexadentate metal ligation environments. Metal detection within a cell might therefore be achieved in the absence of strict discrimination at the level of protein-metal binding. It remains unknown why the higher affinity zinc does not associate with NmtR in vivo. This work highlights the need to identify the chemical form(s) of the labile pool(s) of metals accessible by each metal sensor and indeed other metalloproteins.

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