A Cadmium-Lead-sensing ArsR-SmtB Repressor with Novel Sensory Sites

COMPLEMENTARY METAL DISCRIMINATION BY NMTR AND CMTR IN A COMMON CYTOSOL*

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We report a cadmium- and lead-detecting transcriptional repressor from Mycobacterium tuberculosis designated CmtR. Two genes were co-transcribed with cmtR, one encoding a deduced P1 type ATPase. Purified CmtR bound to the cmt operator-promoter, and repression of transcription was lost after introduction of a stop codon into cmtR. Assays of metal-dependent expression from cmt and nmt operator-promoters established that the metal specificity of CmtR in vivo was perfectly inverted relative to the nickel-cobalt sensor NmtR from the same organism, with CmtR totally insensitive to Co(II) or Ni(II) and NmtR totally insensitive to Cd(II) or Pb(II). Absorption spectroscopy of Cd(II)-, Co(II)-, and Ni(II)-substituted CmtR revealed S absorption bands in, or near, these regions, Cys4, Cys35, Asp79, His81, Asp97, Asp99, Glu105, Glu111, and Glu114, retained both binding to these two proteins. Ni(II)-binding isothermal titrations of CmtR are complex, with KD104 M for site1, three orders of magnitude weaker than KD30 for NmtR. Mixing equimolar apo-NmtR and apo-CmtR with 0.9 equivalents of Cd(II) gave Cd(II)-dependent difference spectra almost identical to Cd(II)3-CmtR. Thus, Cd(II) bound to CmtR in preference to NmtR, whereas the converse was true for Ni(II); this correlates faithfully with and provides a simplistic basis for metal-sensing preferences. In contrast, CmtR and NmtR had similar affinities for Co(II), and alternative explanations for metal-sensitivities of seven distinct representatives are known. SmtB-mediated repression is alleviated by Zn(II) (4), Zn(II) (5), ArsR by As(III), Sb(III), and Bi(III) (6), CadC by Cd(II), Pb(II), and Bi(III) (7–9), CzR by Zn(II) and Co(II) (10, 11), MerR from Streptomyces lividans Hg(II) (12), and most recently, NmtR by Ni(II) and Co(II) (13). Two pairs of metal-binding sites in SmtB homodimers (14) include either ligands associated with a3 helices consistent with residues required for inducer recognition in ArsR (15), or ligands from antiparallel a5 helices supplied by each monomer and including residues required for inducer recognition in SmtB (16). Analogous sites have been modeled and then functionally and/or spectrally defined in the related sensors, with additional ligands derived from the amino terminus of the second monomer (8, 17, 18, 20, 21) contributing to the a3 site in some, a3N, or from a carboxyl-terminal extension, a5C, in one (13). Permutations in the use of these sites for allostery, involving distinct ligand sets in sensors with differing selectivities, has been described as “a theme and variations model” (22). Here we report a new sensor in which the residues required for inducer recognition compose completely original sites.

Metal availability within the host cytosol can also contribute to observed specificities (13). Repression mediated by NmtR from Mycobacterium tuberculosis was alleviated by Ni(II) and Co(II) in mycobacterial cells, but solely by Co(II) when introduced into a cyanobacterium. Nonetheless, intrinsic differences between SmtB and NmtR must allow SmtB to respond to Zn(II) but not Co(II), and NmtR to respond to Co(II) but not Zn(II), when tested in a common cell type. In this case, metal-binding preferences of the two proteins do not provide a simplistic explanation for the observed selectivities. NmtR binds Co(II) and Ni(II) but not Cu(II) or Zn(II), whereas CmtR binds Cd(II) and Pb(II) but not Cu(II) or Zn(II).

Some non-essential and several essential metals form complexes of exceptionally high stability with protein centers containing sulfur and/or nitrogen ligands (reviewed in Ref. 1). For a sub-set of proteins to be occupied by less competitive metals, cells are anticipated to contain negligible freely available copper (2) and mercury (the most competitive ions) but also zinc (3), nickel, cobalt, cadmium, and lead (1). Intracellular metal sensors must detect any surplus ions and modulate expression of genes encoding proteins that expel, sequester, or otherwise detoxify the excess. What molecules sense that some critical metal threshold has been exceeded, and how do they discriminate between the elements?

Genes encoding ArsR-SmtB family DNA-binding, metal-responsive transcriptional repressors occur in many eubacterial and archaeabacterial genomes, and the metal-sensing preferences of seven distinct representatives are known. SmtB-mediated repression is alleviated by Zn(II) (4), Zn(II) (5), ArsR by As(III), Sb(III), and Bi(III) (6), CadC by Cd(II), Pb(II), and Bi(III) (7–9), CzR by Zn(II) and Co(II) (10, 11), MerR from Streptomyces lividans Hg(II) (12), and most recently, NmtR by Ni(II) and Co(II) (13). Two pairs of metal-binding sites in SmtB homodimers (14) include either ligands associated with a3 helices consistent with residues required for inducer recognition in ArsR (15), or ligands from antiparallel a5 helices supplied by each monomer and including residues required for inducer recognition in SmtB (16). Analogous sites have been modeled and then functionally and/or spectrally defined in the related sensors, with additional ligands derived from the amino terminus of the second monomer (8, 17, 18, 20, 21) contributing to the a3 site in some, a3N, or from a carboxyl-terminal extension, a5C, in one (13). Permutations in the use of these sites for allostery, involving distinct ligand sets in sensors with differing selectivities, has been described as “a theme and variations model” (22). Here we report a new sensor in which the residues required for inducer recognition compose completely original sites.

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do support the most obvious explanation for the observed specificities for Cd(II) and Ni(II) but not for Co(II). Site-directed mutagenesis has been used to further define the CmtR residues essential for detecting metals. The chemical nature of the ligands and their spatial arrangement provides further insight into the sub-molecular bases of selective metal detection inside cells.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and DNA Manipulations—**Mycobacterium smegmatis mc^155^ and Mycobacterium bovis BCG (Pasteur) were grown with shaking at 37 °C in LB medium (27) containing 0.05% Tween 80 (v/v) as an aeration medium (28), respectively. *Escherichia coli* strains JM109 (Stratagene), JM101, and BL21(DE3) were grown in LB medium. Cells were transformed to antibiotic resistance as described (27, 29). Standard DNA manipulations were performed according to Sambrook et al. (27). All generated plasmid constructs were checked by sequence analysis.

**RNA Manipulation and Reverse Transcriptase (RT)-PCR—**The cmtR region (Fig. 1A) is conserved with 100% identity in *M. bovis* BCG. Nucleic acids were extracted from *M. bovis* BCG using TRIzol reagent (Invitrogen), treated with DNase I (Stratagene), and used (−0.5 μg) as template for reverse transcription with M-MuLV reverse transcriptase (Stratagene), primer 1 (5'-GAAGCTTACCGGCGGTGGCCCGTG-3' (Fig. 1A), and RNAase block (Stratagene), according to manufacturer’s instructions. A control reaction in which reverse transcription was replaced with H2O was included. The reverse transcriptase-PCR amplification reactions used primers I and II (5'-GTCGAGATGACAGGTAGCTG-3') (Fig. 1A).

**Construction of cmtR-lacZ Fusions, Site-directed Mutagenesis, and β-Galactosidase Assays—** *M. tuberculosis* H37Rv DNA was used as template for PCR with primers III (5'-GAAGATTCGCGCCGCCCAACTATCGTG-3') and IV (5'-GAAGATTTGGTGCCTTCTTACCCAATCGTG-3') and the amplification product containing the cmt operator-promoter and cmtR ligated to pGEM-T prior to subcloning into the ScaI/BamHI site of pEM15 (29). (M) to create a transcriptional fusion with lacZ (Fig. 1C). *S. cerevisiae* (Stratagene) site-directed mutagenesis was subsequently used, according to manufacturer's protocols, to generate derivatives with codon substitutions in *cmtR*: Ser^90→Val in a UAG stop codon; Cys^84→Ser; Cys^85→Ser; Cys^86→Ser; Cys^87→Ser; Cys^88→Ser; Cys^89→Ser; His^90→Thr; Gly^91→Ser; Gly^92→Thr; and His^93→Glu; and Asp^94→Ser, and Asp^95→Glu, Asp^96→Asp, and Asp^97→Asp to Ala. The lacZ fusion constructs were introduced into *M. smegmatis* mc^155^ and transformants selected on LB plates supplemented with 50 μg/ml X-gal (Fig. 1B). A control reaction in which reverse transcriptase reactions were performed as described previously (30) in triplicate on at least three separate occasions. The medium was supplemented with various concentrations of metals (described in individual experiments) for ~20 h immediately prior to assays. The metal salts used were ZnSO4, CuSO4, NiCl2, CdCl2, Fe(III) citrate, Pb(NO3)2, Bi(NO3)3, CuSO4, AgNO3, and HgCl2.

**Expression and Purification of CmtR—**The CmtR coding region was amplified by PCR from *M. tuberculosis* H37Rv DNA using primers V (5'-GAAGATATGCGTGTAAGGTACGTG-3') and VI (5'-GAAGATATGCGTGTAAGGTACGTG-3') and ligated to pGEM-T (Promega) prior to subcloning into the NdeI/BamHI site of pET29a (Novagen). Recombinant protein was expressed in E. coli BL21(DE3), cells lysates were prepared (30), and CmtR was purified by binding to heparin-Sepharose (Amersham Biosciences) with 10 mM HEPES (pH 7.8), 50 mM NaCl, 1 mM EDTA, and 1 mM diethiothreitol, and eluted into the same buffer but containing 400 mM NaCl. Fractionation of pooled eluate was done on Sephadex G-75 (246 ml) and concentrated by re-application to heparin-Sepharose equilibrated with 10 mM HEPES (pH 7.8), 50 mM NaCl, and 1 mM diethiothreitol. Elution into a buffer was done with 400 mM NaCl. Final application and elution was from Sephadex-C25 (Amersham Biosciences) in 10 mM HEPES (pH 7.4) and 150 mM KCl in an anaerobic chamber. A single prominent band of the anticipated size (12.487 kDa) was detected by PAGE, and the concentration of purified protein was calculated from the theoretical molar extinction coefficient at 280 nm of 24200 M−1 cm−1.

**Gel Retardation Assays—**Purified CmtR was incubated for 20 min at 25 °C in 20 mM Tris-HCl (pH 7.8), 1 mM diethiothreitol, 1 mM EDTA, 3% glycerol (v/v), and 0.05 mM spermidine with ~0.2 μM non-specific competitor DNA generated using the T7 primer (5'-TAATACGACTACATAAGGG-3') and primer VII (5'-CAAGCTTAGACATCCAAGCGG-3').
with self-ligated pGEM-T as template and ~0.2 μM P1, P2, P3, or P4 probe DNA (Fig. 1D). The latter were generated by PCR with pGEM-T containing cmtr and the cnt operator-promoter region as template, using primer VIII (5’-GACCCAGGCGGATCC-3’) with primer IX (5’-GGGGATCCATTACG-3’) or primer XI (5’-ATGATCCATTACG-3’) for P1, P2, and P4, respectively, or primer XII (5’TCCATATGACGTGTGAGATG-3’) with primer X for P3. Samples were loaded onto 12% (w/v) polyacrylamide gels (30:1, acrylamide:bisacrylamide) and electrophoresed using 1x TBE (27) as the buffer system. Protein-DNA complexes were visualized by ethidium-bromide staining.

Cd(II), Co(II), and Ni(II) Binding Experiments—Titration of apo-Cmtr with Cd(II), Co(II), and Ni(II) under anaerobic conditions were monitored by UV-visible optical spectroscopy in 10 mM HEPES (pH 7.4) and 150 mM KCl essentially as described for SmtB (24). To monitor Cd(II) binding in competition with NmtR (prepared as described in Ref. 13), 20.8 μM Cmtr, 20.8 μM NmtR, or an equimolar mix of both proteins (total protein, 41.5 μM) were incubated in an anaerobic chamber with 18.7 μM Cd(II), and the optical spectra were recorded. Co(II) competition experiments were performed as above but using 37.4 μM Cmtr with 41.5 μM Cmtr or NmtR, or 83 μM of a 1:1 mix of both proteins.

Structural Model of Cmtr—Cmtr structure was modeled against SmtB (14) using SWISSMODEL. The secondary structure of the carboxyl-terminal extension of Cmtr was predicted using PredictProtein.

RESULTS

Ru1993c, cmtr, and cmtA Are Co-transcribed, and Cmtr Binds to a Common Operator-Promoter Region—The proximity of cmtr, Ru1993c, and cmtA indicates that these three ORFs are co-transcribed (Fig. 1A). Furthermore, similarity of the deduced product of cmtr to members of the ArsR-SmtB family of metal-responsive repressors and of cmtA to metal-transporting P7-type ATPases suggests that the former might bind to the upstream operator-promoter region to regulate their transcription. Reverse transcriptase-PCR was used to detect any polycistronic mRNA containing cmtr, Ru1993c, and cmtA. Total RNA was extracted from M. bovis BCG, treated with DNase I, and used as template for reverse transcription with primer I, designed to anneal to sequences within the cmtA coding region (Fig. 1A). To control for PCR amplification of contaminating DNA, a reaction was performed in which H2O was substituted for reverse transcriptase. Products were used as PCR template with primers I and II, the latter annealing to sequences within the cmtr coding region. A reverse-transcriptase-dependent product of the anticipated size of 432 bp was detected (Fig. 1B).

Purified Cmtr was used in gel retardation assays with a 111-bp DNA fragment, P1, containing part of the cmtr operator-promoter and the 5’ end of cmtr (Fig. 1D). A major retarded complex (and several minor retarded complexes) was formed with increasing concentrations of Cmtr with a concomitant decrease in the amount of free probe but no shift of nonspecific competitor DNA (Fig. 1D), demonstrating specific binding of Cmtr to the 111-bp P1 fragment. To further map the Cmtr-DNA binding site, assays were repeated using truncations of P1. A major complex was detected with P2 and P3 but not with P4 (Fig. 1D), confirming Cmtr binding to sequences contained within a 35-bp DNA fragment that includes a degenerate 10–510 hyphenated-inverted repeat (Fig. 1E).

Cmtr Is a Cadmium- and Lead-responsive Repressor—Cmtr shows the greatest similarity to the Hg(II) sensor MerR from S. lividans, whereas CmtA shows greater similarity to Cd(II)-exporting CadA (31). It is probable that the same metals will be sensed by Cmtr and transported by CmtA. To establish which, if any, metals induce transcription from the cmtr operator-promoter, a 520-bp DNA fragment including the 156-bp cmtr operator-promoter and the entire cmtr coding region was fused to a promoterless lacZ in plasmid pJEM15 (Fig. 1C) and introduced into M. smegmatis mc2155. Elevated β-galactosidase activity was detected in response to exposure (20 h) to maximum permissive concentrations of either Cd(II) or Pb(II) but no other metals (Fig. 2A). In the absence of added metal ions, elevated β-galactosidase activity was detected from an analogous construct in which codon 9 within the cmtr coding region had been converted to a stop codon (Fig. 2B), confirming that Cmtr acts negatively toward expression from the cmtr operator-promoter.

Cd(II)-thiol Bonding in Cmtr—The next challenge was to identify the inducer recognition sites of Cmtr. Comparison with the amino acid sequences of related sensors (Fig. 3A) reveals that Cmtr lacks potential liganding residues that precisely align with α3α2N ligands required for inducer recognition by some ArsR-SmtB family members (8, 15, 18, 20), although Cys35 is proximal to helix α3 and Cys36 is proximal to the amino terminus. Cmtr has only two (Asp79 and His101) potential ligands aligning with the four conserved ligands at predicted α5 regulatory metal-binding sites of SmtB and NmtR (13, 16, 21), although additional candidate ligands are present within a 21-residue carboxyl-terminal extension relative to SmtB, which is predicted (using PredictProtein) to form an additional α-helix not seen in other family members. The modeled structure of Cmtr is based on the coordinates for SmtB (14). Fig. 3B shows a hypothetical Cmtr homodimer.

UV-visible absorption spectroscopy was used to detect any involvement of thiols in Cmtr-Cd(II) binding. S− to Cd(II) ligand-to-metal charge transfer confers distinctive absorption in the UV region of −240 nm (8, 9). Fig. 4 shows spectra from anaerobic titration of Cmtr with Cd(II) giving distinctive Cd(II)-dependent features; the Inset shows the Cd(II) binding isotherm fit to a site with a predicted maximum absorption at 240 nm of 13,900 M−1 cm−1. The absolute magnitude of the molar absorption (ε) at −240 nm reports on the number of Cd-S bonds, with ε240 ~5500–6000 M−1 cm−1 per S−-Cd(II) bond (8). These data are, therefore, consistent with at least two cysteiny1 ligands involved in Cd(II) coordination. Of course, any “non-thiolate” Cd(II)-binding residues which lack ligand-to-metal charge transfer would not be detected by this method. The curve fit (Fig. 4, Inset) implies that saturation of binding requires the addition of more than one equivalent of Cd(II), which is suggestive of a second site.

Identification of Cysteine Residues Essential for Cd(II)-recognition in Vivo—Having established that Cd(II)-coordination in vitro involves thiols, the next question is which, if any, cysteine residues are essential for inducer recognition and might supply these ligands? Cmtr has a total of six cysteine residues (Fig.

![Fig. 2. Cmtr responds to Cd(II) and Pb(II) in a mycobacteria. β-galactosidase activity in mycobacterial cells containing cmtr (A) or the stop codon derivative (B). Cells were grown with no metal supplement and maximum permissive concentrations of Zn(II) (75 μM), Co(II) (0.2 μM), Ni(II) (0.5 μM), Cd(II) (2.5 μM), Pb(II) (5 μM), Bi(III) (0.3 μM), Cu(II) (0.5 mM), Ag(I) (0.5 μM), Fe(III) (50 μM), or Hg(II) (0.01 μM).](http://www.jbc.org/)

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A, and substitution of Cys4, Cys35, Cys57, Cys61, and Cys102 with Ser in CmtR created functional repressors that mediated low expression of lacZ from the cmt operator-promoter in mycobacterial cells grown with no metal supplements (Fig. 5). However, β-galactosidase activity was constitutively elevated in cells containing a Ser substitution of Cys24, which is analogous to that observed after introduction of a stop codon (Fig. 2B), establishing that the Cys24 mutant is repressor non-functional. Most significantly, β-galactosidase activity was not elevated in cells containing Ser substitutions of Cys57, Cys61, and Cys102 at a Cd(II) concentration that causes loss of repression by wild-type CmtR (Fig. 5) and also by the Cys4- and Cys35-substituted mutants (Fig. 5). Consistent with this, alleviation of repression in the presence of maximum permissive [Pb(II)] was only observed for the Cys4- and Cys35-substituted CmtR mutants, not the Cys57, Cys61, and Cys102 mutants (data not shown). These results demonstrate that at least Cys57, Cys61, and Cys102 are obligatory for Cd(II) recognition, with Cys24 (essential for repression) being a potential fourth ligand. It remains to be established whether or not this represents a full complement of ligands, hexadentate Cd(II)-protein complexes are known (19), or indeed whether these residues all contribute toward a single common site, because in ZiaR, two different pairs of sites at α3N and α5C are obligatory for inducer recognition. Most importantly, the combination of Cys residues essential for inducer recognition in CmtR (Fig. 5) do not map to the location of known sites in any other ArsR-SmtB family members (Fig. 3A).

Other Potential Ligands at α5 or α5C Are Not Mandatory for Inducer Recognition—Asp79 and His81 in CmtR align with two
Metal ligands, Asp97, Asp 99, Glu 105, Glu 111, and Glu 114. Perterminal extension of CmtR includes a further five potentials derived from a carboxyl-terminal extension, and the carboxylmore, two ligands required for inducer recognition by NmtR are not require a predictable

Cmtr and NmtR Have Opposing Metal Specificities in the Same Mycobacterial Cell—At maximum permissive concentrations of Cd(II), Pb(II), Ni(II), and Co(II), CmtR "senses" the first two metals but not the last two (Fig. 2A). This finding seems opposite to that for NmtR. However the response of NmtR to Pb(II) has never been tested, and it remains to be established whether NmtR responds to Cd(II) or whether CmtR responds to Co(II) or Ni(II) at any concentration other than the maximum permissive one. Expression from the respective operator-promoter regions was examined in response to a range of concentrations of metals, up to and including inhibitory doses (Fig. 6). At permissive concentrations, Cd(II) is the most potent inducer of expression from the cmt operator-promoter, whereas no viable concentration of Ni(II) or Co(II) alleviates CmtR-mediated repression. In direct contrast, NmtR only responds to Ni(II) and Co(II). Thus, the two sensors have evolved to show the exact converse discrimination between Cd(II) and Pb(II) versus Ni(II) and Co(II) in the common mycobacterial cytosol.

UV-visible Absorption Spectroscopy of Ni(II)- and Co(II)-Cmtr Complexes—Co(II) and Ni(II) UV-visible absorption spectroscopy is commonly used to report on the coordination sphere for spectrally inert metals such as Zn(II) and, to some extent, Cd(II). Analyses of Co(II)- and Ni(II)-Cmtr complexes may also provide clues about the molecular bases of metal selectivity relative to the Ni(II) and Co(II) sensor NmtR. An aerobic titration of Cmtr with Co(II) results in the appearance of intense absorption in the 300–400 nm region (Fig. 7A), with peaks at ≈310 nm and ≈370 nm assignable to S to Co(II) ligand-to-metal charge transfer and, hence, indicative of cysteinyI-coordination for this metal in common with Cd(II) (9, 21, 24). It is noted that the spectral base line rises upon a second addition of Co(II) up to 1 equivalent, and there was visible precipitation above 1.4 equivalents.

Fig. 7B shows the Ni(II) UV-visible absorption spectrum for Cmtr. Similar to Co(II)-Cmtr, there is low absorption in the region assignable to d-d ligand field transitions of Ni(II), which is suggestive of high coordinate Ni(II). There is evidence of S to Ni(II) ligand-to-metal charge transfer (300–450 nm region), with peaks at ≈310 nm and ≈400 nm, implying cysteine coordination, but these only appear after the addition of excess Ni(II). Minimal S to Ni(II) ligand-to-metal charge transfer was detected below two molar equivalents of Ni(II) per Cmtr monomer; absorbance continues to increase with superstoichiometric [Ni(II)] (Fig. 7B, large inset). The Ni(II) binding isotherm generated when absorbance is plotted as a function of [Ni(II)]; [Cmtr monomer] is initially sigmoidal (Fig. 7B, small inset) but is also consistent with multiple Ni(II) sites (Fig. 7B, large inset). A fit for the first site implies weak affinity, with $K_{app} = 1.8 \times 10^4 \text{M}^{-1}$. Slight precipitation was observed with increasing [Ni(II)]; although this did not impair the spectra, it is suggestive of adventitious liganding. Ni(II) seems to form non-native complexes with Cmtr, exploiting coordination geometries involving more ligands than tetrahedral sites.

Cmtr Outcompetes NmtR for Cd(II) but Has Similar in Vitro Affinity for Co(II)—What is the difference between Cmtr and NmtR that inverts the metals they sense? Comparison of SmtB and NmtR showed that discrimination was not caused by selective binding but rather by selective effects on allostery. Is this also true of Cmtr and NmtR, which have evolved in the
same cell? Competitive binding experiments were used to directly test which protein preferentially acquires Cd(II). Cd(II)$_{0.9}$-CmrR has distinctive spectral features between 200 nm and 300 nm, assignable to S$^\text{+}$ to Cd(II) ligand-to-metal charge transfer (Fig. 8A). In contrast, NmrR is devoid of cysteine residues, and Cd(II)$_{0.9}$-NmrR lacks these features. The addition of 0.9 equivalents of Cd(II) to pre-mixed equimolar apo-NmrR and apo-CmrR gave an absorption spectrum similar to that obtained with Cd(II)$_{0.9}$-CmrR but unlike that obtained with Cd(II)$_{0.9}$-NmrR (Fig. 8A). Thus, Cd(II) binds to CmrR in preference to NmrR, implying a difference in affinity of at least one order of magnitude.

No viable dose of Ni(II) or Co(II) alleviated CmrR-mediated repression in the mycobacterium, despite these metal ions acting as potent inducers of NmrR (Fig. 6). Consistent with this, Ni(II) had a far lower apparent affinity for CmrR than for NmrR, with $K_{\text{app}}$ site$_1$ of CmrR = $1.8 \times 10^4$ M$^{-1}$ (Fig. 7B, Inset), whereas $K_{\text{Ni}}$ for NmrR $\geq 2 \times 10^7$ M$^{-1}$ (13). However, in a competitive binding experiment to examine Co(II) binding, the maximum absorption of the distinctive spectral features of Co(II)$_{0.9}$-CmrR were reduced by half in the presence of an equimolar amount of NmrR (Fig. 8D), implying that these proteins have very closely matched affinities for Co(II). Hence, the metal specificities of NmrR and CmrR in vivo can only partly be explained by their relative metal affinities in vitro.

**DISCUSSION**

CmrR is a newly identified DNA-binding (Fig. 1D) Cd(II)- and Pb(II)-sensing SmtB-ArsR transcriptional repressor (Fig. 2) that functions in the same cytosol as the related Ni(II) and Co(II) sensor, NmrR. Metal discrimination was completely inverted; CmrR failed to respond in vivo to any viable concentration of Ni(II) or Co(II), and NmrR failed to respond to any viable concentration of Cd(II) or Pb(II) (Fig. 6). Purified CmrR formed specific complexes in vitro with a 35-bp region of a shared cmrR-cmrA operator-promoter containing a 10–510 region of dyadic symmetry (Fig. 1D). Expression of $\beta$-galactosidase activity from the shared operator-promoter was repressed in mycobacterial cells containing functional cmrR but was elevated when a stop codon was introduced within the cmrR ORF (Fig. 2). The locations of the metal-sensing sites in CmrR are totally unlike those of any other family member (Figs. 3 and 5). Affinities of NmrR and CmrR for Co(II) were closely matched in vitro, and alternative factors must facilitate this facet of metal-selectivity, whereas discriminatory sensing of Cd(II) and Ni(II) wholly correlated with the relative affinities of the two proteins; CmrR had the higher affinity for Cd(II) (Fig. 8) and NmrR (13) had the higher affinity for Ni(II) (Fig. 7).

The effector-binding sites of all previously characterized SmtB-ArsR sensors are (i) at the $\alpha$3-helix associated with DNA-binding plus (generally) additional ligands from the amino terminus of the second monomer, and/or (ii) at antiparallel $\alpha$5-helices. Although two potential ligands of the $\alpha$5 sites are perfectly conserved in CmrR (Fig. 3), they are not obligatory for inducer recognition (Fig. 5), and neither are Asp (two) nor Glu residues (three) from an extended carboxyl terminus obligatory (Figs. 3 and 5). It remains possible that CmrR has “vestigial” secondary Cd(II) binding sites involving these residues but, crucially, it is unlike NmrR, which has $\alpha$5C “sensory” sites. Cys$^4$ and Cys$^{30}$ could be modeled to contribute to variant $\alpha$3N sites, but the thiolate groups of these residues are also not required for Cd(II) recognition in vivo (Fig. 5). CmrR is, therefore, unlike the related Cd(II) and Pb(II) sensor, CadC, which has $\alpha$3N sensory sites (8, 9, 18, 20). In CadC, the $\alpha$3N sites are composed exclusively of four Cys residues providing some preference for thiophilic Cd(II). CmrR absorption spectra similarly reveal Cd(II)-thiolate coordination with at least two Cys per site (Fig. 4). To locate the atypical sensory sites, all six Cys were converted to Ser and three (Cys$^{47}$, Cys$^{61}$, and Cys$^{102}$) were required for inducer recognition, while a fourth was repression non-competent and hence cannot be excluded as a potential ligand (Fig. 5). The proximity and spacing of Cys$^{57}$ and Cys$^{61}$ (three intervening residues places them in a similar orientation on the predicted $\alpha$R-helix) strongly argues in favor of these thiols contributing to a common site. Clearly, additional structural information is required to resolve the most unusual conformation of the CmrR-inducer recognition site(s), but present best predictions support four candidate Cys ligands derived from deduced $\alpha2$, $\alpha R$, and predicted $\alpha$6 helices.

CmrR “sensed” when some critical thresholds of the non-essential metals Cd(II) and Pb(II) were exceeded in a mycobacterial cell, leading to de-repression of genes encoding proteins presumed to expel these ions, but NmrR was totally unresponsive. NmrR detected vital thresholds of Ni(II) and Co(II), whereas CmrR completely failed to respond to these ions (Fig. 6). How is such perfect discrimination between elements achieved? For two of the metals, Cd(II) and Pb(II), the most naive explanation holds true. At equilibrium in vitro, $K_{\text{Ni}}$ for NmrR is at least three orders of magnitude higher than for CmrR (Fig. 7B; Ref. 13), whereas Cd(II) associates with CmrR in preference to NmrR after direct in vitro competition (Fig. 8A). Thus, these metals could partition to the correct sensors inside a cell based upon relative metal-binding preferences. The inducer recognition sites of NmrR lack any Cys residues consistent with a Cd(II) preference for the thiolate sites of CmrR (Figs. 4 and 5). Six ligands are available in the metal coordination sphere of NmrR (13, 23), which is ideal for metals such as Ni(II) that prefer a higher coordination number. A lack of an obvious absorbance peak attributable to d-d transitions in
Ni(II)-CmtR spectra (Fig. 7B) indicates that Ni(II) also recruits more than four ligands from CmtR, but the low affinity, the complexity of the isothermal titration curve, and the evidence of precipitation suggest that this is a non-native site. In summary, physical and chemical properties of the metal-sensing sites of these two proteins match measured metal-binding preferences which, in turn, correlate with observed intracellular metal discrimination for two of the respective metals.

Unlike Cd(II) and Ni(II), the selective detection of Co(II) can not be explained by observed metal-binding preferences. Direct competition shows that Co(II)-affinities are very similar for CmtR and NmtR in vitro (Fig. 8). Selective detection by NmtR of metals that favor a higher coordination number is not based upon selective binding but upon a demand for extra ligands to be recruited into the binding site to mediate the conformation changes that reduce the stability of DNA-protein complexes somehow discern different inorganic elements in expression under excess metal that are similar, and yet they explain the apparent discrepancy between relative affinities and inducer specificities.

Evolution has generated cell detectors that trigger gene expression under excess metal that are similar, and yet they somehow discern different inorganic elements in M. tuberculosis. The inherent preferences of Cd(II) for thiol ligands and of Ni(II) for higher coordination numbers have selected a thiol-containing site in the Cd(II)- and Pb(II)-sensing CmtR but not NmtR, an octahedral site in the Ni(II)- and Co(II)-sensing NmtR. This contributes toward preferential binding of at least two of the detected metals to the correct sensors. Cd(II) to CmtR and Ni(II) to NmtR, but Co(II) partitions equally in vitro. We already know that Ni(II) and Co(II) sensing by NmtR further demands formation of high coordination number complexes to trigger allostery, thereby avoiding gene expression by adventitious Zn(II)-NmtR complexes that only exploit four ligands. We remain intrigued by the possibility that CmtR also has “secondary metal-selection checkpoints” operating at the level of allostery. Pb(II) and Cd(II) have relatively large ionic radii. Perhaps the unusual sensory site of CmtR, with ligands derived from disparate structural elements, demands that only metals capable of forming associations with larger coordination spheres de-repress gene expression.

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A Cadmium-Lead-sensing ArsR-SmtB Repressor with Novel Sensory Sites: COMPLEMENTARY METAL DISCRIMINATION BY NMTR AND CMTR IN A COMMON CYTOSOL

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