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Biochemical and biophysical characterization of the manganese transport regulator (MntR) from Bacillus subtilis

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Biochemical and biophysical characterization of the manganese transport regulator (MntR) from Bacillus subtilis

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Chemistry

by

Misha Golynskiy

Committee in charge

Professor Seth Cohen, Chair
Professor Partho Ghosh
Professor Patricia Jennings
Professor William Trogler
Professor Virgil Woods, Jr.

2007
The dissertation of Misha Golynskiy is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2007
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<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>alpha</td>
</tr>
<tr>
<td>Å</td>
<td>angstrom, $10^{-10}$ meters</td>
</tr>
<tr>
<td>a.a.</td>
<td>amino acid</td>
</tr>
<tr>
<td>ANS</td>
<td>8-anilino-1-naphthalenesulfonic acid</td>
</tr>
<tr>
<td>AntR</td>
<td>anthracis repressor</td>
</tr>
<tr>
<td>ArsR</td>
<td>arsenic regulator</td>
</tr>
<tr>
<td>AUC</td>
<td>analytical ultra centrifugation</td>
</tr>
<tr>
<td>$\beta$</td>
<td>beta</td>
</tr>
<tr>
<td>$^2$H</td>
<td>deuterium</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis(2-nitrobenzoic acid), also known as Ellman’s reagent</td>
</tr>
<tr>
<td>DtxR</td>
<td>diphtheria toxin repressor</td>
</tr>
<tr>
<td>DXMS</td>
<td>deuterium exchange mass spectrometry</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>Fur</td>
<td>ferric uptake regulator</td>
</tr>
<tr>
<td>GuHCl</td>
<td>guanidine hydrochloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>$N$-(2-hydroxyethyl)piperazine-$N'$-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HTH</td>
<td>helix-turn-helix</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>inductively coupled plasma optical emission spectroscopy</td>
</tr>
</tbody>
</table>
IdeR       iron-dependent regulator
IPTG       isopropyl β-D-thiogalactopyranoside
M          molar, mol L⁻¹
Mag-fura-2  2-[2-(5-carboxy)oxazole]-5-hydroxy-6-aminobenzofuran-N,N,O-triacetic acid
MALDI-TOF  Matrix Assisted Laser Desorption Time-of-Flight mass spectrometer
MntR       manganese transport regulator
MS         mass spectrometry
NRAMP      Natural Resistance Associated Macrophage Protein
PAGE       polyacrylamide gel electrophoresis
ref.       reference
RMS        root mean square
RT         room temperature
SE         size exclusion
SH3        Src homology 3-like domain
SmtB       Synechococcus metallothionein locus, gene B
SVD        single value decomposition
TFA        trifluoroacetic acid
Tₘ         melting temperature
Tris       tris(hydroxymethyl)aminomethane
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The text of chapter 3, in full, is a reprint of the material as it appears in the journal *Biochemistry*, 2006, vol. 45(51):15359-15372 “Metal Binding Studies and EPR
Spectroscopy of the Manganese Transport Regulator MntR”. The dissertation author was the primary researcher and/or author and the co-authors (Gunderson WA, Hendrich MP, Cohen SM) listed in this publication contributed to or supervised the research which forms the basis for this chapter.

The text of chapter 4, in full, is a reprint of the material published in the *Journal of Biological Inorganic Chemistry*, 2007 “Conformational studies of the manganese transport regulator (MntR) from *Bacillus subtilis* using deuterium exchange mass spectrometry”. The dissertation author was the primary researcher and/or author and the co-authors (Li S, Woods V Jr, Cohen SM) listed in this publication contributed to or supervised the research which forms the basis for this chapter.

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M.V. Golynskiy “DNA-binding and mechanistic insights into MntR, a manganese regulatory protein” Oral Presentation, Graduate Research Symposium in Bioinorganic Chemistry, Ventura, California, January 2005


PUBLICATIONS


Peters R.G., Golynskiy M.V., Baughman R.G. Tetracarbonylbis[(pentafluoroethyl)diphenylphosphine] chromium(0). Acta Cryst. (2002); E58, m70-m71

Metals ions are employed in biology for several reasons including their ability to participate in redox chemistry, catalysis, and structural stabilization of proteins. However, the properties that make metal ions so widely utilized in biology can be potentially hazardous, particularly if abnormal quantities of these ions are accumulated. This necessitates a mechanism by which the balance between uptake of essential metal ions and efflux of excess essential or toxic metal ions, otherwise referred to as metal homeostasis, can be maintained. Bacteria employ a unique set of metal responsive transcription factors (metalloregulators) to manage this delicate balance.
The biochemical and biophysical characterization of MntR, a manganese responsive regulator from the DtxR family is the focus of this thesis. Fluorescence anisotropy was used to probe the DNA-binding of wild type MntR, MntR D8M, and MntR E99C mutants to the cognate DNA recognition sequences \( mntH \) and \( mntA \) in the presence of various divalent metal ions. Our studies demonstrate the extent to which these metal ions are able to activate MntR to bind DNA. In addition, these studies shed light on the origin of metal specificity between MntR and DtxR and are in agreement with \textit{in vivo} data reported in the literature.

In addition to investigating the DNA-binding abilities of MntR, we also examined the metal binding affinities of this protein in order explain how it fits into the DtxR family and the general field of metalloregulatory proteins. The results demonstrate that MntR metal-binding affinities loosely follow the Irving-Williams series. Interestingly, the protein exhibits the weakest affinity for one of its cognate metal ions.

Finally, the metal-mediated mechanism of DNA binding by MntR was studied. Initial investigations using circular dichroism and an environmentally-sensitive dye ANS showed that metal binding stabilizes either tertiary or quaternary structure of MntR. Subsequent studies focused on localizing these structural changes using deuterium exchange mass spectrometry (DXMS) and demonstrated that metal-binding serves to rigidify the pre-organized structure of MntR. Moreover, contrary to typical observation of transcription factors, cofactor (metal) binding does not appear to alter the structure of helix-turn-helix DNA-binding motif.
Chapter 1.

Metal Ion Homeostasis and Metalloregulatory Proteins
METAL ION HOMEOSTASIS

Introduction

Metal ions are widely employed in a variety of cellular functions and are vital for survival of both prokaryotes and eukaryotes. While group 1 and 2 metals such as Na⁺/K⁺ and Ca²⁺/Mg²⁺ are some of the most biologically abundant metal ions, it is the transition metal ions (predominantly Zn, Cu, Ni, Co, Fe, Mn, Mo and W) that are utilized in essential processes such as respiration, photosynthesis, and other processes important to cellular metabolism (1, 2). In particular, due to their electronic configurations, transition metal ions are able to participate in catalysis and redox chemistry within proteins or to serve as anchors for structural stabilization. Unfortunately, the reactivity of these metal ions, if left unregulated, can be damaging to the cell in numerous ways (unregulated redox chemistry, generation of reactive oxygen species) (3). This presents a challenge for cells, as metal ion concentrations need to be maintained within a specific range necessary for growth and biological activity (4). By employing a variety of metal-binding and transporting proteins, metal ions are taken up into the cell, delivered to the correct metalloproteins, and effluxed, thus modulating metal homeostasis, or the delicate balance of metal concentrations in the cell.

Molecules Involved in Metal Homeostasis

Several types of biomolecules are involved in maintaining metal ion homeostasis. In bacteria, the task of metal ion acquisition is performed by siderophores and a variety of metal transporter proteins, with siderophores being more prominent and
better understood in comparison (5-8). Siderophores are small molecules that chelate iron and form very thermodynamically stable metal complexes. These chelators readily retrieve iron(III) from the environment, with iron-siderophore uptake facilitated by complex-specific receptors (5). Other essential metal ions are brought in and out of the cell through membrane-embedded transporter proteins (6, 9).

Several class of membrane-embedded transporters are involved in the metal homeostasis of the cell. A number of cations are translocated in and out of the cell using NRAMP-like (Natural Resistance Associated Macrophage Protein-like) proteins, members of the P-type ATPase, or ATP-binding cassette (ABC) transporter superfamilies (6, 7, 10). While NRAMP-like proteins generally employ H⁺ gradients, P-type ATPases and ABC transporters employ ATP hydrolysis as the energy source for ion transport (6, 7, 10, 11). Further, NRAMP-like proteins and P-type ATPases only transport the metal ions by themselves (7, 8, 11, 12). In contrast, members of the ABC transporter superfamily can transport metal ions into the cell either by themselves, as hydrated metal complexes or, as non-specific small molecule chelator complexes (e.g. citrate) (5, 6, 10, 13). For the purpose of this introduction, “membrane-embedded transporter” will be used as a general term for any member of transporter families mentioned above.

Despite supplying the cell with the necessary metal ions, both methods of metal uptake present complications for growth and biological activity. Utilization of siderophores places an energetic and material demand on the organism as their synthesis necessitates production of a myriad of proteins and enzymes to build, transport, and process these molecules (5). Membrane-embedded transporters constantly transfer
metal ions and are not always very selective for their cognate metal ion (9). Furthermore, some of the membrane-embedded transporters can transport toxic metal ions into the cell due to their similarities in ionic size and coordination preferences to biologically relevant metal ions (9). Thus, in addition to maintaining essential metal ion homeostasis, it is also important to limit cells from synthesizing superfluous proteins or acquiring potentially toxic metal ions.

Besides the proteins involved in metal influx and efflux, further molecular machinery may be employed to modulate the intracellular trafficking of metal ions. In particular, metallochaperones mediate metal shuttling and delivery between proteins, inserting the appropriate metal ion into correct proteins (1, 14).

**Metalloregulators**

The behavior of siderophores and the membrane-embedded transporters described above necessitates a mechanism by which the bacteria can maintain metal ion homeostasis and limit the expenditure of cellular resources (5, 15). This level of control is exerted using metal-responsive transcription factors commonly referred to as metalloregulatory proteins, or metalloregulators. In particular, metalloregulators interact with the operons of genes involved in, but not limited to, metal homeostasis. This interaction can result in transcription activation or repression, depending on the metal ion concentrations found in the cell and the type of metalloregulatory protein. Ultimately, one can view metalloregulators as metal sensor proteins that allow the bacteria to respond to the metal concentrations inside the cell.
Metalloregulatory proteins can be classified as either activators or repressors depending on whether they activate or repress the transcription of genes under regulation. This classification can be further specified as either positive or negative to indicate whether metal binding results in the gain or loss of the activation/repression function. For example, a positive repressor refers to a protein that represses gene transcription in presence of the cognate metal ion whereas a negative repressor suppresses transcription of the gene in the absence of the cognate metal ion (Figure 1.1). The various modes of regulation allow the cell to effectively manage metal homeostasis while efficiently using cellular resources. For example, in case of metal ion uptake, expression of proteins involved in metal ion transport is necessary until the cellular metal demands have been met. This is distinct from metal efflux mechanisms, where membrane-embedded transporters do not need to be expressed until the cell encounters either toxic metal ions or starts to over-accumulate essential metals (9).
Figure 1.1. Schematic diagram of positive (A) and negative (B) repression. Metal-free and metal-bound regulator is illustrated in blue and red, respectively. RNA polymerase subunits that interact with DNA are illustrated as red and green ovals; DNA shown as overlapping blue and green curves.

METALLOREGULATOR FAMILIES

A number of metalloregulatory systems are known and depending on the system, can control the expression of up to 90 genes (6, 16-18). These genes are typically involved in metal transport or utilization of the regulating metal ion, although other proteins may be regulated as well. For example, it has been observed that some members of DtxR family also regulate toxin production in response to metal concentration of the cell (6, 19-23). To date, five main families of metalloregulators
have been described (2). Two of these families are largely responsible for handling either efflux of excess essential metal ions or of toxic metal ions, while the other three are mainly involved in the acquisition of essential metal ions (2, 6, 19-25) (Table 1.1). A brief introduction to these families is provided below to facilitate better understanding of the field of metalloregulatory proteins.
Table 1.1. A brief overview of metalloregulatory families. Shown are some of the characterized proteins within a particular family, their native metal ion(s), and the purposed function of the metalloregulator.

<table>
<thead>
<tr>
<th>Family</th>
<th>Protein</th>
<th>Cognate Metal(s)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MerR</td>
<td>MerR</td>
<td>Hg$^{2+}$</td>
<td>Toxic</td>
</tr>
<tr>
<td></td>
<td>CadR</td>
<td>Cd$^{2+}$, Zn$^{2+}$, Pb$^{2+}$</td>
<td>Metal Efflux</td>
</tr>
<tr>
<td></td>
<td>ZntR</td>
<td>Zn$^{2+}$, Pb$^{2+}$, Cd$^{2+}$</td>
<td>Excess Essential</td>
</tr>
<tr>
<td></td>
<td>CueR</td>
<td>Cu$^+$, Ag$^+$, Au$^+$</td>
<td>Metal Efflux</td>
</tr>
<tr>
<td></td>
<td>CoaR</td>
<td>Co$^{2+}$</td>
<td></td>
</tr>
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<td>Zn$^{2+}$, Co$^{2+}$</td>
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<td>Zn$^{2+}$, Co$^{2+}$</td>
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<td></td>
<td>SmtB</td>
<td>Ni$^{2+}$, Co$^{2+}$</td>
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<td>ArsR</td>
<td>ArsR</td>
<td>As$^{3+}$, Sb$^{3+}$</td>
<td>Toxic</td>
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<tr>
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<td>Cd$^{2+}$, Pb$^{2+}$, Bi$^{3+}$, Zn$^{2+}$</td>
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<td>TroR</td>
<td>Mn$^{2+}$, Zn$^{2+}$</td>
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<td>SirR</td>
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MerR Family

MerR, the prototypical member of this family and the first reported metalloregulatory protein is a Hg$^{2+}$-responsive regulator (22, 24). Apo-MerR is both an activator of its own synthesis and a repressor of a series proteins involved in mercury resistance; upon sensing Hg$^{2+}$, MerR initiates transcription of these proteins (22, 24). At the time of its discovery, MerR was the first transcription factor for which the apo (repressor) and holo (activator) forms of the protein were bound to the same DNA site (22). The MerR family is perhaps one of the more remarkable within the metalloregulatory field as it can be further classified into MerR (Hg-responsive), metal-responsive MerR-like (responds to metal ions other than Hg), and MerR-associated (typically responsive to either oxygen or low molecular weight molecules) regulators (22). Analysis of 156 bacterial genomes suggests that within the MerR family, 5% are MerR, 44% are metal-responsive MerR-like, and 51% are MerR-associated regulators (22). Other metal-responsive members of this family (most notably CueR and ZntR) are also involved in regulation of toxic or potentially toxic metals (Table 1.1), and regulate transcription of metal-specific ATPases in response to changes in metal ion concentrations (22, 24, 26, 27).

MerR family members tend to share an N-terminal helix-turn-helix DNA-binding domain, but differ within the C-terminal inducer domain, where the metal ions bind in the case of MerR and metal-responsive MerR-like regulators (Figure 1.2) (22). In general, MerR family proteins are homodimers, which bind a symmetric DNA dyad sequence separated by 19 or 20 nucleotide spacer. Metal-responsive members of this family bind up to 2 metal ions per dimer, employing primarily Cys ligands to chelate
the metal ions in linear or trigonal planar geometry (2, 28). An exception to this observation is Zn-ZntR which employs Cys and His ligands and forms a binuclear metal site (22). MerR itself is unique in that it recruits two Cys ligands from one monomer and one Cys ligand from another (29). Members of MerR family known to date appear to exhibit very high (sub-picomolar) metal affinities suggesting that they are designed to deal with hypersensitive metal stress (28, 30). In vitro and in presence of thiols, the mer promoter responds to 90% of induced activity at $\sim 10^{-8}$ M Hg$^{2+}$ (31).

Biophysical data suggests that apo-MerR binds to a promoter DNA already occupied to RNA polymerase, forming a ternary complex between MerR, the DNA promoter and RNA polymerase (24). MerR distorts the DNA, preventing transcription of the genes until metal binding occurs. This event reorganizes the DNA promoter to be linear, allowing for transcription to proceed (24). An interesting application of protein engineering where the metal-binding inducer domains of MerR and ZntR were swapped demonstrated that the DNA-binding ability of MerR proteins can be regulated by various inducer domains (26). This finding suggested that the diversity of the MerR proteins likely resulted from an evolutionary response due to “modular” protein design. Specifically, it was proposed that the DNA encoding for various inducer domains was fused with DNA encoding for DNA-binding function, creating a new protein that bound DNA upon interaction with its inducer (26).

The overall mechanism of action of MerR appears conserved within the family, as a similar mechanism of action has been suggested for ZntR and CueR proteins (32). While crystallographic evidence has proven elusive for MerR, crystal structures of CueR and ZntR have been reported (Figure 1.2) (28).
Figure 1.2. Crystal structure of Cu\(^{+}\)-CueR (top, PDB ID: 1Q05). One monomer is colored gray, another colored in gradient from N-terminus (red) to C-terminus (blue). Metal ions are displayed as orange spheres; coordinating atoms are shown in yellow (S atoms from Cys), blue (N atoms from His) and red (O atoms from PO\(_4\)). Metal site of Cu\(^{+}\)-CueR (bottom left, PDB ID: 1Q05) and Zn\(^{2+}\)-ZntR (bottom right, PDB ID: 1Q09), with bonds illustrated as dashed blue lines.

**ArsR Family**

This class of metalloregulators, named after the prototypical member ArsR, also responds to toxic metal ion and excess metal ion concentrations, to some extent
overlapping with the metal ions regulated by the MerR family (Table 1.1). The ArsR family members tend to upregulate transcription of metal transporting ATPases upon sensing their cognate metal ions in the cellular environment (23, 33-38). Similar to the MerR family, ArsR proteins interact with DNA as homodimers, binding a conserved 12-2-12 inverted repeat using the DNA-binding helix-turn-helix motif located in the N-terminus of the protein. Unlike the MerR family members, however, ArsR class utilizes up to two distinct metal sites per protein monomer (Figure 1.3). Further, in case of ArsR family, while the dimeric apo protein is DNA bound and prevents transcription of the genes, no ternary complex with RNA polymerase is observed (18, 23).

As already noted, most ArsR proteins have two distinct metal sensing sites, although metal sensing typically necessitates binding to only one. One of the sites (α3N) binds metal ions in 3- or 4-coordinate environment with predominantly sulfur-based ligands and, responds to soft, toxic/heavy metal ions. The other site (α5) is composed largely of nitrogen- and oxygen-based ligands generating 4- to 6-coordinate environments and responds to harder metal ions (23). The metal binding of ArsR proteins is weaker than those encountered with the MerR family, generally with affinities ($K_d$) in the $10^{-8}$ to $10^{-12}$ M range (18, 23, 33, 34, 37, 39, 40).
Figure 1.3 Crystal structure of Zn\textsuperscript{2+}-SmtB (top, PDB ID: 1R23). One monomer is colored gray, another colored in gradient from N-terminus (red) to C-terminus (blue). Zn\textsuperscript{2+} is displayed as orange sphere; coordinating atoms are shown in blue (N atoms from His) and red (O atoms from Asp and Glu). Approximate locations of residues involved formation of $\alpha$3N site is highlighted in green on the gray monomer. $\alpha$5 metal site of Zn\textsuperscript{2+}-SmtB (bottom) with bonds illustrated as dashed blue lines.

Several mechanisms of action have been observed for the ArsR family; these have been primarily for proteins that bind metals with the $\alpha$5, as opposed to $\alpha$3N site. In the reported mechanisms, metal binding at the $\alpha$5 site can cause the protein to either contract away from the DNA or to freeze out protein conformations that favorably interact with DNA (35). Apo- SmtB binds its cognate operator sequence with
nanomolar affinity and it is has been suggested that the structural changes that occur upon metal-binding of the ArsR family of proteins result in significantly lower DNA binding affinities (~500-2000 fold decrease), allowing the protein to dissociate from the DNA (18, 23).

**NikR Family**

One of the recently discovered family of metalloregulatory proteins is the NikR family involved in Ni metabolism (41). NikR family members have been observed in *E. coli* and *H. pylori*, where incorporation of Ni into urease and [Ni-Fe]-hydrogenase genes is essential for metabolic adaptation to the surroundings (42). NikR is a positive repressor that down-regulates transcription of membrane-embedded transporter (NikABCDE) in response to intracellular Ni$^{2+}$ concentrations. Interestingly, to date NikR is the only observed metalloregulator that functions as a tetramer (a dimer of dimers), typically binding one metal ion per subunit (42). The NikR monomer consists of an N-terminal DNA-binding ribbon-helix-helix motif that is connected to a metal binding domain via an α-helix (41, 42). Binding of a single metal ion to NikR activates this protein to bind a 28 base pair palindromic operator (6 base pair DNA recognition sequences are separated by 16 nucleotide spacer), with nanomolar affinity; DNA binding is improved upon additional metal ion binding (43, 44). The first metal site is created at the interface between two NikR subunits; the metal site has a square planar geometry, with one monomer donating two His and one Cys ligand and another donating one His chelating residue (45). With the exception of Mn$^{2+}$, this site binds rather tightly to first row transition metal ions ($K_d$ $10^{-17}$ to $10^{-9}$ M), binding Ni$^{2+}$ with
picomolar affinity (43). This observation implies that Ni uptake, in contrast to Fe and Mn (vide infra), is very tightly regulated. The second metal site is located between the DNA-binding ribon-helix-helix motif and the metal binding motif (42). Such positioning suggests that binding in this site helps rigidify the NikR structure and reduce mobility of the DNA-binding domain. The identity of the second metal ion is still a subject of debate as solution studies suggest that the largest enhancement in DNA affinity is achieved by additional Ni$^{2+}$ binding, while crystallographic work suggests that this role can be performed by K$^+$ (42, 44).

Several crystal structures of NikR have been determined, allowing for comparison between the apo, holo, and DNA-bound isoforms (Figure 1.4) (42, 45). The different isoforms are unique, suggesting a multi-step mechanism of activation for DNA-binding. Initial metal binding reorganizes several of the $\alpha$-helices in the metal-binding domain, exposing one of them to interact with DNA. This causes localization of the protein near the DNA (via electrostatic interactions), positioning the DNA-binding motif near the DNA-binding site and giving secondary structure to the helix connecting the DNA-binding and metal-binding motifs (42). Interestingly, the structure of the DNA-binding motif itself is not altered by the metal binding event (42). Following the initial DNA binding, the second metal binding event likely serves to reduce the mobility of the DNA-binding motif as it recruits ligands from both DNA-binding and metal-binding motifs. In particular, this event would position the DNA-binding domains to interact even more favorably with the DNA (42).
Figure 1.4. Crystal structure of Ni$^{2+}$-NikR (top, PDB ID: 2HZV). One of the interacting dimers is shown green, the other is multicolored by monomers. Within the colored dimer, one monomer is colored gray, another is colored in gradient from N-terminus (red) to C-terminus (blue). Ni$^{2+}$ and K$^+$ are displayed as orange and green spheres, respectively; coordinating atoms are shown in yellow (S atoms from Cys), blue (N atoms from His), and red (O atoms from Asp and Glu). Metal sites of NikR, Ni$^{2+}$-NikR (bottom left) and K$^+$-NikR (bottom right); bonds illustrated as dashed blue lines.
Fur Family

Ferric uptake regulator (Fur) is a very abundant (up to 10000 copies per cell) iron sensor protein involved in regulation of over forty genes and is commonly found in Gram-negative bacteria (5, 20, 25, 46). Similar to NikR, Fur functions as a positive repressor, largely regulating transcription of proteins involved in siderophore synthesis and transport (25). Furthermore, it was recently discovered that Fur is also regulated by oxidative stress response, potentially minimizing the harmful effects generated by Fe$^{2+}$-mediated Fenton chemistry (47). In the cell, Fur is a metal dependent homodimer with two metal sites per monomer (48, 49). The apo-protein exhibits no DNA-binding activity, while metal ion binding results in nanomolar DNA-binding affinities (50). Similar to the other metalloregulatory proteins discussed so far, the Fur protein can be loosely classified into two domains. The N-terminal contains the DNA-binding helix-turn-helix motif, while the two metal sites (one is structural, the other is regulatory) are located in the C-terminal of the protein (51). In vivo, the structural site of Fur is Zn$^{2+}$-bound and has tetrahedral geometry while the regulatory site is Fe$^{2+}$-bound and has distorted octahedral geometry. In the Fur crystal structure from P. aeruginosa, both the structural and the regulatory site coordinate metal ions using 2 His and 2 Glu residues, albeit in different coordination geometries (51). However, in E. coli Fur, which is more homologous with the majority of known Fur proteins, the structural site is thought to employ two Cys, and either His and Asp/Glu, or two Asp/Glu ligand (52). In vitro, transition metal ions other than Fe$^{2+}$ can bind and activate Fur for binding the palindromic DNA consensus sequence known as the “Fur box” (52). Biochemical studies suggest that the structural site possesses a high affinity for Zn$^{2+}$, although actual
$K_d$ has not been determined (53, 54). The metal binding affinities in the regulatory site are significantly lower, in the $10^{-9}$ to $10^{-5}$ M $K_d$ range (53, 54). Metal binding in the regulatory site loosely follows the Irving-Williams series, binding tighter to late first row transition metal ions (53, 54).

To date, one crystal structure of Fur from *P. aeruginosa* has been determined; additionally, crystallographic and solution studies on fragments of *E. coli* Fur have been performed (Figure 1.5) (51, 52, 55). The crystal structure from *P. aeruginosa* is not truly representative of Fur proteins observed in most bacterial species as the Cys residues typically involved in metal coordination are not conserved. Deuterium exchange mass spectrometry (DXMS) solution studies on Mn$^{2+}$-bound *E. coli* Fur suggest that metal binding by Fur results in exposure of the DNA-binding helices, rendering them capable of interacting with the cognate DNA (55). Structural studies suggest that in solution the N-terminal DNA-binding motif is well organized while the metal binding C-terminus is disordered (52). Zn$^{2+}$-binding at the structural site results in protein dimerization, while binding of iron at the regulatory site is thought to somehow result in structural rearrangement and organization of the N-terminal $\alpha$-helix that plays a significant role in DNA interactions (52). Other members of the Fur family have been reported and include non-iron responsive members involved in the regulation of Mn, Ni, and Zn, although structural and biophysical data on these proteins is limited (56-58).
Figure 1.5 Crystal structure of Zn$^{2+}$-Fur from *P. aeruginosa* (top, PDB ID: 1MZB). One monomer is colored gray, another colored in gradient from N-terminus (red) to C-terminus (blue). Metal ions are displayed as orange spheres; coordinating atoms are shown in blue (N atoms from His) and red (O atoms from Glu). Metal site of Zn$^{2+}$-Fur (bottom), with bonds illustrated as dashed blue lines.
DtxR Family

In contrast to utilization of the Fur protein in Gram-negative bacteria, Gram-positive bacteria utilize a structurally different and yet functionally homologous protein for iron regulation. Diphtheria toxin repressor (DtxR, isolated from *Corynebacterium diphtheriae*) along with iron-dependent regulator (IdeR, isolated from *Mycobacterium tuberculosis*) comprise the prototypical members of the DtxR family that is primarily involved in regulation genes involved in iron acquisition, transport, utilization, and toxin production. Diphtheria toxin repressor (DtxR) was first isolated as a repressor of the diphtheria toxin production in *C. dyphtheriae* (59). It has been implicated in bacterial pathogenesis, as under iron-limiting conditions, diphtheria toxin production is upregulated to kill the host, releasing iron for further bacterial acquisition (59). DtxR forms a metal dependent homodimer with two metal binding sites per monomer and binds to DNA using a helix-turn-helix motif (Figure 1.6) (60). DtxR has been classified as consisting of an N-terminal domain that incorporates the DNA-binding motif and metal-binding dimerization core of the protein and a C-terminal SH3 domain (60, 61).

*In vivo* DtxR is selectively responsive to iron(II), while *in vitro* other metal ions can activate it for DNA binding (62). The metal binding sites are positioned ~9 Å apart and serve unique functions (62-64). The primary site is 6-coordinate with a mixed nitrogen-, oxygen-, and sulfur-based ligand set and is thought to be the sensory site while the ancillary site is 5-coordinate with a nitrogen- and oxygen-based ligand set and is thought to be a structural site (63, 65). *In vitro*, the metal binding affinities (K_d) tend to fall in the 10^{-7} to 10^{-4} M range, with higher affinities exhibited for the structural/ancillary site and weaker affinities for the regulatory/primary site (63, 64).
Upon metal ion binding, DtxR/IdeR undergoes structural changes and metal-bound protein can bind cognate DNA recognition sequences with $K_d \sim 10$-1000 nM (66, 67).

**Figure 1.6.** Crystal structure of DNA bound Co$^{2+}$-IdeR (top, PDB ID: 1U8R). One monomer is colored gray, another colored in gradient from N-terminus (red) to C-terminus (blue). Metal ions are displayed as orange spheres; coordinating atoms are shown in yellow (S atoms from Cys and Met), blue (N atoms from His) and red (O atoms from Glu and backbone of Cys). Metal site of Co$^{2+}$-IdeR (bottom; coordinating H$_2$O from the primary site which completes the octahedral geometry was not found in this structure), with bonds illustrated as dashed blue lines.
While early biophysical and crystallographic evidence identified some structural features of DtxR, the proposed mechanism of action remained controversial and was not determined until recently (60, 61, 63, 68). Solution studies suggest that the N-terminal of DtxR is largely disordered in the apo form and becomes reorganized upon metal binding. The metal binding acts so as to promote protein dimerization and to favorably position the DNA binding helices of the HTH motif for interacting with DNA while the SH3 domain serves to stabilize the apo form of the protein (61, 69).

**General Observations Regarding Metalloregulatory Families**

By comparing members of the various metalloregulatory families it becomes apparent that the ArsR and MerR family members are typically negative regulators and frequently possess metal binding affinities in or significantly below the nanomolar range (22-24, 26, 28, 33, 70). In contrast, Fur and DtxR family members discovered to date are positive regulators with affinities in the nanomolar to sub-millimolar range suggesting that in regards to uptake of Fe, the organism either needs or can tolerate higher concentrations of this ion (54, 58, 63). One possible advantage to such differences in the metal-binding affinities between positive and negative regulators may be to allow bacteria to be more sensitive to changes in toxic metal ions concentrations compared to essential metal ions.

In addition to using metal binding affinities to discriminate between various metal ions, metalloregulatory proteins can also discriminate between metal ions by employing sets of specific ligands (2). In particular, amino acids with sulfur or mixed
sulfur/nitrogen donor atoms are more likely to be found chelating larger and softer metal ions while nitrogen/oxygen donors are more typical for smaller and harder metal ions (2). The differences in metal coordination not only lead to different metal selectivity between metalloregulator families but, with the exception of NikR proteins, also results in loosened metal specificity between family members (Table 1.1) (2, 22, 24, 34, 54, 58). For the topic at hand it should be noted that the different metalloregulatory classes have different mechanisms of action as well as different metal coordination and affinities.

MANGANESE HOMEOSTASIS

Cellular Requirements for Manganese

Significant interest in the field of manganese regulation and homeostasis was sparked by the observation that bacteria possess a homolog of the eukaryotic natural resistance associated macrophage protein (NRAMP) (21). NRAMP proteins are employed by the host in response to bacterial pathogenesis (71, 72). In particular, NRAMP proteins transport divalent metal ions, most commonly Fe^{2+} and Mn^{2+} (72). In case of bacterial invasion, the host limits access to these ions prior to using oxidative stress to kill the pathogen (71-73). The presence of an NRAMP homolog in bacteria suggested that this protein may function to counteract the host’s attempt to limit nutrients (21). Further, this homolog was found to be primarily involved in manganese and cadmium transport and thus was classified as the proton-dependent manganese
transporter, (MntH) in bacteria (11, 12, 21). Interestingly, manganese can be utilized either directly or as a cofactor by superoxide dismutases (MnSOD) to combat oxidative stress, implicating this metal in some aspects of bacterial pathogenesis (21). Another implication of manganese in pathogenesis was noted by discovery of the Lyme disease pathogen Borrelia burgdorferi, whose genome entirely lacked iron requiring proteins (74). The odds for survival of such an organism in a limited nutrient environment are likely to be substantially higher than those of Fe²⁺-requiring bacteria (74). These observations suggested that there had to be a pathway to maintain bacterial Mn²⁺ homeostasis; indeed in 2000 Helmann et al. first isolated and described a manganese transport regulator from the DtxR family (75).

**MntR**

The initial discovery of MntR found it to be responsible for Cd²⁺/Mn²⁺ regulation in B. subtilis by transcribing the mntH and mntABCD genes (75). Since then, MntR homologs have been found in a number of bacterial species, in some cases regulating one or both of the transporter protein mentioned above (mntABCD is sometimes referred to as sitABCD in different organisms) (76-78). These genes encode Mn²⁺ transporting proteins that either use H⁺ coupled gradient or ATP hydrolysis as an energy source to facilitate metal ion transfer through the cell membrane. The proposed scheme for metal ion regulation by MntR suggests that when the manganese requirements of the cell are met, MntR binds Mn²⁺, resulting in DNA binding and repression of genes responsible for encoding the aforementioned manganese transporter proteins. Presumably, when manganese levels drop below those required for B. subtilis,
the metal ions dissociate from MntR, and MntR is released from the mnt operons allowing for transcription of the transporter proteins.

In contrast to 226 amino acid long DtxR, MntR is only 142 amino acids long as it lacks the C-terminal SH3 domain found in DtxR. Sequence alignment between MntR and DtxR suggested that MntR shares ~30% sequence homology with DtxR, although alignment of only the first 130 amino acids (without the SH3 domain) increases the homology to ~70%. Interestingly, sequence alignment between these two proteins suggested that the metal chelating residues from the primary (sensing) site of DtxR are largely conserved. However, instead of employing the sulfur-based ligands found in DtxR (Met10 and Cys102), MntR utilizes oxygen-based ones (Asp8 and Glu99) (Figure 1.6 and 1.7). *In vivo* mutant studies introduced sulfur-based ligands into MntR and oxygen-based ligands into DtxR in order to probe whether the difference in the activation profiles was due to differences between metal-chelating residues (79). In particular, M10D, C102E and M10D/C102E DtxR mutants were compared with D8M, E99C and D8M/E99C MntR mutants in their ability to respond to iron and manganese in vivo (79). These studies were able to demonstrate that replacing sulfur-based ligands with oxygen-based ligands in DtxR loosens Fe$^{2+}$ specificity and indeed makes DtxR more Mn$^{2+}$-responsive (79). The “reverse” switch, wherein the oxygen-based MntR ligands were replaced with sulfur-based ones, did not result in a change of the MntR metal specificity, although some mutations loosened the metal specificity enough to elicit comparable activation by both Fe$^{2+}$ and Mn$^{2+}$ ions (79).

Initial *in vitro* characterization from our lab had shown that in contrast to DtxR/IdeR proteins, MntR exists as a metal-independent homodimer in solution (80).
These studies also showed that MntR binds to the \textit{mntH} sequence with $10^{-9}$ to $10^{-6}$ M affinities ($K_d$), depending on the nature of transition metal ion bound to MntR (80). Native metal ions, Mn$^{2+}$ and Cd$^{2+}$, were found to be the most potent activators of DNA binding (80). Crystallographic studies showed that MntR forms a homodimer with two metal ions per protein monomer (Figure 1.7); however, the MntR metal binding sites form a single binuclear cluster in contrast to two distinct sites observed in DtxR (81, 82). Two different crystallographic structures of Mn$^{2+}$-bound MntR have been reported, with slightly different coordination environments in the metal-binding site (81, 82). The differences between the two structures have been attributed to the temperature at which the crystallographic data were collected (binuclear AB site at 100K with three bridging ligands vs binuclear AC site at 298K with two bridging ligands). Further, the Cd$^{2+}$- and Ca$^{2+}$-bound MntR structures have been reported (82). These structures also display the AC geometry observed in Mn$^{2+}$-MntR structure collected at 298K; therefore the AC site was assigned as the physiologically relevant site. Lastly, structures for Zn$^{2+}$-MntR and Mn$^{2+}$-MntR D8M mutant have been reported, both of which form mononuclear metal sites, implicating formation of the binuclear site as a key step for strong activation of DNA binding (81, 82).
Figure 1.7. Mn$^{2+}$-bound MntR (top, PDB ID 2F5F). One monomer shown in gray, the other in color gradient from N-terminus (red) to C-terminus (blue). Metal ions are displayed as orange spheres; coordinating atoms are shown in blue (N atoms from His) and red (O atoms from Asp and Glu). Metal site (AC) of Mn$^{2+}$-MntR (bottom), with bonds illustrated as dashed blue lines. H$_2$O molecules are depicted as small red balls.

**Purpose of the Investigation**

The goal of this thesis is to ultimately address how MntR functions *in vivo*. Through our investigations, we want to better understand the mechanism of MntR action, how it fits into the DtxR family, and its physiological relevance for *B. subtilis*. 
For example, the cell first must have enough free metal available for sensing, a condition that is dependent on the metal-binding affinities of MntR. If that requirement is fulfilled and MntR can “sense” the activating metal ion, the protein needs to be able to selectively respond to the appropriate cognate metal ion and discriminate against it from the pool of intracellularly available metal ions. The selection of appropriate metal ions is likely to be governed by the ability of MntR to form either the most thermodynamically stable or the most kinetically inert complexes with a given metal ion. Finally, the activating metal ion needs to be bound with the appropriate coordination stoichiometry and geometry in order to invoke the allosteric structural changes in MntR. This structural change is ultimately translated into the DNA-binding response of MntR, allowing bacteria to respond to the metal concentration of the cognate metal ion.

At the time of its discovery, MntR was one of the few non-iron responsive members of the DtxR family. The differences in the metal-activation profile of MntR from prototypical DtxR proteins, as well as its ability to respond to both essential and toxic metal ions pose interesting questions. Studying these aspects of MntR behavior will improve our understanding of how MntR fits into the iron responsive DtxR family of metalloregulatory proteins and why it responds to toxic metal ions such as Cd\(^{2+}\). Furthermore, characterizing the metal-activation profile, metal-specificity, and mechanism of action of MntR will give more insight into the general behavior of metalloregulatory proteins.
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Chapter 2.

Metal-Induced Structural Organization and Stabilization of the Metalloregulatory Protein MntR
ABSTRACT

MntR is a metalloregulatory protein that helps to modulate the level of manganese in *Bacillus subtilis*. MntR shows a metal-response profile distinct from other members of the DtxR family of metalloregulatory proteins, which are generally considered to be iron(II)-activated. As part of an ongoing effort to elucidate the mechanism and metal-selectivity of MntR, several biophysical studies on wild type MntR and two active site mutants, MntR E99C and MntR D8M have been performed. Using circular dichroism (CD) spectroscopy, the thermal stability of these proteins has been examined in the presence of various divalent metal ions. Fluorescence intensity measurements of 8-anilino-1-naphthalenesulfonic acid (ANS) were monitored to examine the folding of these proteins in the presence of different metal ions. These experiments indicate that MntR undergoes a significant conformational change upon metal binding that results in stabilization of the protein structure. These studies also show that the MntR D8M active site mutation causes a detrimental effect on the metal-responsiveness of this protein. Fluorescence anisotropy experiments have been performed to quantify the extent of metal-activated DNA binding by these proteins to two different cognate recognition sequences. Binding of MntR and MntR E99C to the *mntA* cognate sequence closely parallels that of the *mntH* operator, confirming that the proteins bind both sequences with comparable affinity depending on the activating metal ion. Fluorescence anisotropy experiments on MntR D8M indicate significantly impaired DNA binding, providing additional evidence that MntR D8M is a dysfunctional regulator.
INTRODUCTION

MntR is one of a small number of manganese(II)-responsive members of the large DtxR family (of which DtxR and IdeR are prototypical members) of metalloregulatory proteins, the majority of which are iron(II)-responsive systems (1-3). Other proteins in the DtxR family that have been assigned as potential manganese(II) sensors include ScaR, SirR, and TroR (4-7); all of these proteins are potentially useful candidates for elucidating how iron(II)- and manganese(II)-activated DtxR proteins discriminate between these ions.

Earlier studies have demonstrated that MntR has a low affinity for the \textit{mntH} regulatory sequence in the absence of transition metal ions (8, 9). In the presence of divalent manganese or cadmium, MntR is strongly activated and demonstrates nanomolar affinity for the \textit{mntH} consensus sequence (10). Oligomerization studies show no change in the quaternary structure of MntR in the apo or holo forms, revealing MntR to be a stable homodimer in solution (10). The crystal structure of MntR (Figure 2.1) shows that the monomer subunits interact through a substantial \(\alpha\)-helical region (Asp123 to Lys136) at the C-terminal end of the polypeptides, which may explain the tight self-association of MntR (11). There are notable differences between the structure of MntR and other structurally characterized members of the DtxR family (Figure 2.1) of metalloregulatory transcription factors (11-14). DtxR, the prototypical iron(II)-responsive member of this family found in \textit{Corynebacterium diphtheriae}, is described as consisting of three domains: an N-terminal DNA-binding helix-turn-helix motif, a metal-binding dimerization interface, and a C-terminal SH3-like domain (15).
However, recent NMR data suggests alternative DtxR assignment with the N-terminal domain consisting of both the DNA-binding motif and the metal-binding dimerization interface of DtxR (16). In contrast to the original domain classification of DtxR, MntR only has two major domains, a DNA-binding and a metal-binding dimerization domain.

**Figure 2.1.** Crystal structures of Co$^{2+}$-IdeR (left, PDB ID: 1U8R) and Mn$^{2+}$-MntR (right, PDB ID: 2F5F). Common structural features are highlighted as follows: DNA-binding winged helix-turn-helix motif is highlighted in red, the dimerization interface in blue and the α-helix connecting these functionalities is highlighted in green. Structure differences between the two protein are highlighted as follows: C-terminal α-helix of MntR is shown in purple; C-terminal SH3 domain of IdeR is shown in orange. Co and Mn atoms are shown as magenta and yellow spheres, respectively.

The most significant structural differences between DtxR and MntR from a bioinorganic view point are found in the metal binding site. DtxR and MntR are both found to bind two metal ions per protein monomer; however, the structure and positioning of these binding sites are notably different (Figure 2.2). The most obvious difference between the metal binding sites is that DtxR has two distinct binding sites (M$^{2+}$⋯M$^{2+}$ ~9 Å) while MntR is found to have a single binuclear site (Mn$^{2+}$⋯Mn$^{2+}$ 4.4 Å), with the metal ions bridged by two protein side chains and an aquo/hydroxo ligand (15, 17-19). Sequence alignment of the two proteins shows that many of the ligating
residues are conserved. The MntR active site recruits ligands generally conserved from the primary site (site ‘C’ in Figure 2.2) of DtxR, with only His77 from the ancillary site (site ‘A’ in Figure 2.2). Two water molecules are also found in the binuclear core of MntR.

Figure 2.2. Stick depiction of the metal-binding sites of Co$^{2+}$-IdeR (top; H$_2$O molecule that completes octahedral coordination of site C is not found in this structure), Mn$^{2+}$-MntR (bottom left) and Mn$^{2+}$-MntR D8M (bottom right). Metal sites are labeled A (ancillary site in DtxR/ IdeR) and C (primary site in DtxR/ IdeR). Sulfur, oxygen and nitrogen atoms are colored yellow, red and blue, respectively, with H$_2$O molecules depicted as red spheres. Bonds from coordinating atoms are shown as dashed blue lines.
The crystal structure of the mutant MntR D8M bound to Mn\(^{2+}\) has also been determined and demonstrates that the binuclear metal site is no longer present. Both Met8 and Glu99 are twisted away from their corresponding locations in the wild type holoprotein, which results in an active site with only a single bound metal ion in a different coordination environment. The single Mn\(^{2+}\) ion in MntR D8M is coordinated by Glu11, His77, Glu102, and a water molecule in the ‘A’ site of the protein (Figure 2.2). Despite the changes in the metal binding site of MntR D8M, the overall protein fold is nearly identical to that of wild type MntR (11).

Recent experiments have sought to address the issue of differential metal ion specificity in DtxR and MntR by mutagenesis and heterologous expression studies (9). These investigations demonstrated that both protein selectivity and the cellular environment contribute to the overall behavior in vivo. Interestingly, although mutation of key active site residues could convert DtxR from an iron(II)- to a manganese(II)-responsive repressor (DtxR M10D/C102E), mutagenesis of one (MntR D8M, MntR E99C) or both of the analogous MntR sites (MntR D8M/E99C) did not generate an iron(II)-selective response. In vivo, MntR E99C showed no ability to respond to Fe\(^{2+}\), while MntR D8M was activated by Fe\(^{2+}\) to a slightly lesser degree than that obtained with Mn\(^{2+}\) (9). Why these MntR active site mutants failed to acquire Fe\(^{2+}\) sensitivity has not been resolved.

In an attempt to understand the effects of active site mutations on the metal-selective activation of MntR, this chapter details several in vitro experiments on wild type MntR and two of the previously reported active site mutants, MntR E99C and MntR D8M. Data presented in this study show that the active site changes in MntR
E99C produce measurable, but not large differences in DNA-binding metal activation profiles, consistent with in vivo findings; however, several pieces of evidence are provided that show the behavior of MntR D8M is notably different from the wild type protein. These studies also shed light on the mechanism of MntR activation. Metal complexation is found to have a significant effect on MntR stability and structure, consistent with an allosteric mechanism whereby the MntR structure is reorganized and optimized for DNA recognition at the tertiary level upon metal binding.
RESULTS

**Preparation and Characterization of MntR E99C and MntR D8M**

The MntR E99C and MntR D8M mutants were characterized by MALDI-TOF-MS, analytical sedimentation equilibrium ultracentrifugation, size-exclusion (SE) chromatography, and circular dichroism (CD) spectroscopy. Analytical sedimentation ultracentrifugation absorbance data were fit to a single, idealized species (Figure 2.3). Apparent molecular weights for MntR E99C and MntR D8M were 35284±1351 and 33529±1723, in good agreement to the expected values for protein homodimers of 33438 and 33522, respectively.

**Figure 2.3.** Ultracentrifugation data for apo MntR E99C (left) and MntR D8M (right) monitored by absorbance at 280 nm. The line through the data is a nonlinear least-squares fit from which the apparent molecular weight was obtained. [MntR E99C] = 15 μM; [MntR D8M] = 20 μM. Speed = 35,000 rpm. Buffer = 20 mM HEPES, pH 7.2 @4 °C, 200 mM NaCl, 5% (v/v) glycerol.

In addition, evaluation by size-exclusion chromatography was consistent with the proteins eluting as homodimers with apparent molecular weights in the range of 33-
35 kDa (data not shown). The proteins were characterized by MALDI-TOF mass spectrometry; MntR E99C generated two peaks at \( m/z \) 16745 Da [M+Na]\(^+\) (calculated 16742 Da) and 16610 Da [M-Met+Na]\(^+\), the latter peak characteristic of the protein with the N-terminal methionine residue truncated (calculated 16611). MntR D8M generated a single peak at \( m/z \) 16648 Da [M-Met+Na]\(^+\) (calculated 16653), indicating that the peak corresponds to MntR D8M with the N-terminal methionine residue truncated. Analysis of MntR E99C with Ellman’s reagent (DTNB) gave a value of 0.88±0.12 free thiols per monomer (1 free thiol expected), consistent with at least ~90% of the protein being in the properly reduced form. CD spectroscopy of MntR, MntR E99C, and MntR D8M in sodium phosphate buffer showed the expected features at 208 and 222 nm (Figure 2.4) characteristic of a highly \( \alpha \)-helical secondary structure \((10, 11, 17)\). As found with wild type MntR \((10)\), addition of Mn\(^{2+}\) to MntR E99C or MntR D8M (data not shown) did not produce significant changes in the 222 nm feature in the CD spectrum \((23)\).
Figure 2.4. CD spectrum of apo MntR (filled circles), MntR E99C (×), and MntR D8M (open squares) in sodium phosphate buffer. The expected double minima are observed for each protein. The calculated percent α-helicity for wild type MntR is ~52%, somewhat less than the ~65% α-helicity found in the crystal structure of MntR. Slightly lower values were obtained for MntR D8M (50%) and MntR E99C (~47%). [Protein] = 8 μM. Buffer = 50 mM Na₂HPO₄, pH 7.5 @RT., 200 mM NaCl. Path length = 2 mm. T = 25 ºC.

Thermal Denaturation Studies Using Circular Dichroism Spectroscopy

Binding of Mn²⁺ does not significantly alter the secondary structure composition of these proteins. As a means to determine what, if any effect, metal binding had on the structure of MntR, thermal denaturation experiments were performed on these proteins in the apo and holo forms. Stabilization of protein structure upon metal binding has been found to be important for the metalloregulatory protein NikR (24). The thermal melting of the proteins was followed by CD spectroscopy at the maximal ellipticity of 222 nm. These studies were performed in the protein storage buffer instead of the
sodium phosphate buffer to minimize precipitation of metal phosphate complexes (Figure 2.5).

**Figure 2.5.** CD spectra of apo MntR (left) in sodium phosphate buffer (filled circles) and sodium phosphate plus 20 mM HEPES buffer (open circles). Although the feature at 208 nm is obscured by the HEPES buffer the feature at 222 nm remains unperturbed. Thermal denaturation curves (right) for apo MntR monitored by CD spectroscopy at 222 nm in 50 mM sodium phosphate buffer (filled circles) and 20 mM HEPES buffer (open circles). The data show that the denaturation behavior is identical in both buffers and that the absorbance of HEPES at 222 nm is negligible and does not interfere with the denaturation experiment. [Protein] = 9 μM. Sodium Phosphate Buffer = 50 mM Na₂HPO₄, pH 7.5 @ RT, 200 mM NaCl. HEPES Buffer = 20 mM HEPES, pH 7.2 @ 4 °C, 200 mM NaCl, 5% (v/v) glycerol. Path length = 2 mm.

Heating of MntR at a rate of 1 °C per minute resulted in a clear melting transition and loss of the CD signal (Figure 2.6). Visible inspection of the sample upon complete denaturation revealed precipitation of MntR and consistent with this observation, the protein could not be refolded upon cooling (CD signal did not return upon cooling, data not shown). The folding is not reversible, therefore, the system is not under equilibrium
conditions (22); however, the midpoint of the denaturation curve was reproducible and a melting temperature ($T_M$) of $\sim 67$ °C was obtained for apo MntR (Table 2.1).

Figure 2.6. Thermal denaturation curves monitored by CD spectroscopy at 222 nm for wild-type MntR (top), MntR E99C (bottom left) and MntR D8M (bottom right). Unfolding of apo protein (open squares) and of protein in the presence of 1.0 mM Mg$^{2+}$ (open diamonds), Ca$^{2+}$ (open circles), Mn$^{2+}$ (filled squares), Co$^{2+}$ (filled diamonds), Ni$^{2+}$ (filled circles), and Cd$^{2+}$ (×) are shown. Data for MntR E99C in the presence of Cd$^{2+}$ is not provided because no unfolding transition was observed up to 100 °C. [Protein] = 8 μM. Buffer = 20 mM HEPES, pH 7.2 @4 °C, 200 mM NaCl, 5% (v/v) glycerol. Path length = 2 mm.
Table 2.1. Melting transition temperatures \( (T_M) \) for MntR proteins obtained from circular dichroism measurements during thermal denaturation in the presence of various divalent metal ions. All values are based on an average of at least two independent experiments. All values are reproducible to \( \pm 1 \) °C.

<table>
<thead>
<tr>
<th>Metal Ion (1 mM)</th>
<th>MntR (°C)</th>
<th>MntR E99C (°C)</th>
<th>MntR D8M (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo (no metal)</td>
<td>67</td>
<td>73</td>
<td>68</td>
</tr>
<tr>
<td>( \text{Mg}^{2+} )</td>
<td>67</td>
<td>72</td>
<td>67</td>
</tr>
<tr>
<td>( \text{Ca}^{2+} )</td>
<td>68</td>
<td>72</td>
<td>67</td>
</tr>
<tr>
<td>( \text{Mn}^{2+} )</td>
<td>83</td>
<td>77</td>
<td>76</td>
</tr>
<tr>
<td>( \text{Co}^{2+} )</td>
<td>86</td>
<td>89</td>
<td>85</td>
</tr>
<tr>
<td>( \text{Ni}^{2+} )</td>
<td>81</td>
<td>87</td>
<td>76</td>
</tr>
<tr>
<td>( \text{Cd}^{2+} )</td>
<td>&gt;90(^a)</td>
<td>&gt;100(^a)</td>
<td>84</td>
</tr>
</tbody>
</table>

\(^a\) Complete denaturation was not observed up to 100 °C and a satisfactory endpoint could not be obtained.

Addition of 1.0 mM \( \text{Mn}^{2+} \) to the solution resulted in a significant thermal stabilization of MntR, with a \( T_M \) of \( \sim 83 \) °C. Similar increases were found in the presence of \( \text{Co}^{2+} \) and \( \text{Ni}^{2+} \), but not upon addition of \( \text{Mg}^{2+} \) or \( \text{Ca}^{2+} \), demonstrating that the observed stabilization is specific to activation by transition metal ions \( (10) \). Addition of 1.0 mM \( \text{Cd}^{2+} \) resulted in enhanced stabilization of the protein with only partial melting observed above 90 °C. Thermal denaturation experiments with MntR E99C were generally comparable to that found with wild type protein (Table 2.1), with two notable exceptions. First, in the presence of 1.0 mM \( \text{Mn}^{2+} \), the melting transition of MntR E99C showed biphasic behavior (Figure 2.6); the origin of this biphasic behavior is presently unknown. Second, addition of 1.0 mM \( \text{Cd}^{2+} \) stabilizes MntR E99C such that no melting transition is observed up to 100 °C. In contrast, MntR D8M showed
significant changes in thermal stabilization behavior relative to wild type MntR (Table 2.1). MntR D8M was far less stabilized by the presence of 1.0 mM Mn$^{2+}$, with only a ~8 °C increase in $T_M$ compared to a ~16 °C increase with wild type MntR. The stabilization obtained with Ni$^{2+}$ is also reduced relative to wild type; moreover, unlike wild type MntR or MntR E99C, MntR D8M undergoes a melting transition at ~84 °C in the presence of 1.0 mM Cd$^{2+}$ (Figure 2.6). These thermal denaturation studies were the first indicators that MntR D8M has notably different behavior from the other proteins investigated here.

**Conformation Changes Monitored by ANS Fluorescence**

The data obtained from thermal denaturation CD experiments indicated that activating metal ions were able to stabilize the structure of wild type MntR. To gain further insight into the nature of this stabilization, fluorescence studies with 8-anilino-1-naphthalenesulfonic acid (ANS) were performed. ANS is a fluorescent chromophore known to bind to hydrophobic surfaces of proteins. Binding of ANS to hydrophobic surfaces increases its fluorescence intensity, while exclusion from such surfaces (i.e. upon protein folding or structural rearrangement) results in a decrease in fluorescence emission (22, 25). Therefore, ANS can be used to monitor changes in the tertiary structure (hydrophobic packing) of proteins. ANS has been successfully used to examine the solution structure of DtxR (16). The combination of 5 μM apo MntR and 500 μM ANS solution results in a ~54% increase in fluorescence intensity versus ANS alone (Figure 2.7). Subsequent addition of 0.1, 0.5, and 1.0 mM Mn$^{2+}$ results in a
reduction of fluorescence intensity to 34%, 15%, and 12%, respectively, above that of ANS alone.

Figure 2.7. ANS fluorescence spectra in the presence of wild type MntR and variable amounts of Mn$^{2+}$. The solid line is the initial spectra of ANS (500 μM) without addition of protein or metal ion. Other spectra are upon addition of: 5 μM MntR (dotted line), 5 μM MntR and 0.1 mM Mn$^{2+}$ (narrow dashed line), 5 μM MntR and 0.5 mM Mn$^{2+}$ (dotted-dashed line), 5 μM MntR and 1.0 mM Mn$^{2+}$ (wide dashed line). $\lambda_{ex}$ = 403 nm. Buffer = 20 mM HEPES, pH 7.2 @4 °C, 200 mM NaCl, 5% (v/v) glycerol. $T$ = 25 °C.

The loss in fluorescence intensity is not due to quenching from the metal ion, as exposure of ANS to 1.0 mM Mn$^{2+}$ in the absence of MntR has no effect on fluorescence emission (data not shown). Therefore, the observed fluorescence decreases are indicative of changes in protein structure. Figure 2.8 summarizes the data for MntR, MntR E99C, and MntR D8M in the presence of ANS and Mn$^{2+}$, Cd$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ at concentrations of 0, 0.1, 0.5, and 1.0 mM. Mg$^{2+}$ and Ca$^{2+}$ showed no significant affect
on ANS fluorescence (data not shown), consistent with the inability of these metal ions to activate or stabilize MntR.

![Figure 2.8](image)

**Figure 2.8.** Changes in ANS fluorescence intensity (507 nm) in the presence of MntR proteins. Plots are labeled for MntR (top), MntR E99C (bottom left), and MntR D8M (bottom right). Bars represent addition of 0 (black), 0.1 (single slash), 0.5 (gray), and 1.0 (crossed slash) mM of the metal ion listed. The column labeled Apo contained ANS and protein only diluted with an equivalent volume of metal-free buffer. All intensities have been background corrected by subtracting the fluorescence intensity of a solution containing an identical concentration of ANS in buffer alone. All samples are the results of at least two independent experiments. \( \lambda_{ex} = 403 \text{ nm.} \) Buffer = 20 mM HEPES, pH 7.2 @ 4 °C, 200 mM NaCl, 5% (v/v) glycerol. \( T = 25 \degree C. \)
By comparison, MntR appears to undergo a complete structural rearrangement in the presence of 0.1 mM Co$^{2+}$ or Cd$^{2+}$, as indicated by a large drop in fluorescence intensity; there were no further substantive changes upon addition of higher concentrations of these metal ions. MntR requires slightly higher concentrations of Mn$^{2+}$ (~0.5 mM) to achieve saturation. Ni$^{2+}$ fails to significantly reduce ANS fluorescence even at concentrations of 1.0 mM. In contrast, MntR E99C shows complete fluorescence reduction, upon addition of 0.1 mM Ni$^{2+}$, Co$^{2+}$, or Cd$^{2+}$, but requires the presence of 1.0 mM Mn$^{2+}$ to achieve the same effect. The increased ability of Ni$^{2+}$ to activate MntR E99C relative to wild type MntR is in agreement with data obtained from fluorescence anisotropy DNA binding studies (vide supra).

Consistent with thermal denaturation experiments presented above, the ANS fluorescence experiments suggest that MntR D8M is structurally and functionally deficient. Although MntR D8M appears to fold in the presence of Co$^{2+}$, the response to Mn$^{2+}$ is significantly reduced. Furthermore, addition of 0.1 mM Cd$^{2+}$ results in an initial reduction in fluorescence intensity, followed by protein precipitation at higher concentrations (manifested as an increase in fluorescence intensity due to scattered light, Figure 2.8). Like wild type MntR, MntR D8M shows little change in ANS fluorescence in the presence of excess Ni$^{2+}$. The ANS data indicate that MntR D8M either does not undergo a substantial structural change upon binding certain metal ions, or that it is perhaps deficient in its abilities to bind these ions.
DNA Binding of MntR

Fluorescence anisotropy studies of MntR and the mntH recognition sequence have been previously used to quantify DNA binding affinity in the presence of various metal ions (10, 26). In an effort to expand upon these earlier results, the binding of MntR to dsmntH26 was studied in the presence of Co$^{2+}$ and Fe$^{2+}$, which were not examined in our earlier report. $K_d$ values were obtained by fitting the fluorescence anisotropy data to a simple 1:1 binding isotherm (10), and were in good agreement with the experimental data. The data indicate that both Co$^{2+}$ and Fe$^{2+}$ are intermediate level activators of MntR, better than Ni$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$, but less effective than Mn$^{2+}$ and Cd$^{2+}$ (Table 2.2). In contrast to in vivo studies (9), these experiments show that MntR can be activated by Fe$^{2+}$; however, the present experiments are performed in the presence of a large excess of metal ion. Binding to dsmntH26 was sequence specific, as a 500-fold excess calf thymus DNA did not interfere with binding of manganese(II)-activated MntR (data not shown). Binding of MntR to the mntA sequence was investigated by using a labeled, 26-base pair oligonucleotide (dsmntA26) containing the core recognition sequence 5'-6F-GATAATTTTGCATGAGGGAAACTTTC-3'. The mntA sequence is the operator sequence in B. subtilis that controls expression of the ABC transporter mntABCD (8). As shown in Table 2.3, MntR binds dsmntA26 in the presence of either 1.0 mM Mn$^{2+}$ or Cd$^{2+}$ with only slightly weaker affinity than found for dsmntH26. The intermediate (Co$^{2+}$ and Fe$^{2+}$) and weak activators (Ni$^{2+}$) stimulate MntR to bind the mntA sequence with slightly improved affinity than found with mntH, but the overall trend is essentially unchanged with Cd$^{2+}$ ≈ Mn$^{2+}$ > Fe$^{2+}$ ≈ Co$^{2+}$ >> Ni$^{2+}$ >> apo. The binding of MntR E99C was also studied using the dsmntH26 and
dsmntA26 oligonucleotides (Figure 2.9). Surprisingly, the affinity of MntR E99C for both oligonucleotides was essentially unchanged from that found for wild type MntR in the presence of Mn$^{2+}$, Fe$^{2+}$, and Cd$^{2+}$ (Tables 2.2 and 2.3). This data is consistent with the earlier finding that both MntR and MntR E99C did not respond to Fe$^{2+}$ in vivo (9). The DNA binding ability in the presence of Co$^{2+}$ and Ni$^{2+}$ improved between 3- to 8-fold over wild type for both sequences. As expected, control titrations with MntR E99C in the presence of 1.0 mM Ca$^{2+}$ and Mg$^{2+}$ showed no significant DNA binding to either sequence (data not shown).

Lastly, fluorescence anisotropy studies of MntR D8M clearly showed the deficient function of this active site mutant. Activation of MntR D8M by all transition metal ions showed reduced affinities for both cognate sequences (Tables 2.2 and 2.3), with a greater impact on the affinity toward dsmntH26. For example, the affinity of MntR D8M for dsmntH26 in the presence of 1.0 mM Mn$^{2+}$ was reduced by more than 10-fold relative to wild type (Figure 2.9).

Although the overall metal selectivity appeared similar to wild type MntR (vide supra), MntR D8M was clearly deficient in DNA binding relative to the other proteins examined. The fluorescence anisotropy data for MntR D8M provide additional evidence that this mutation causes a substantial change in the protein that manifests as both structural and functional anomalies.
Table 2.2. Binding of MntR, MntR E99C, and MntR D8M proteins to the mntH recognition sequence. Dissociation constants ($K_d$) for modeling the fluorescence anisotropy data with a 1:1 binding isotherm. All values are based on an average of at least two independent experiments.

<table>
<thead>
<tr>
<th>Metal Ion (1 mM)</th>
<th>MntR $K_d$ (nM) $^a$</th>
<th>MntR E99C $K_d$ (nM) $^a$</th>
<th>$K_{d-w.t.}/K_{d-E99C}$</th>
<th>MntR D8M $K_d$ (nM) $^a$</th>
<th>$K_{d-w.t.}/K_{d-D8M}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo</td>
<td>&gt;8000 $^b,c$</td>
<td>&gt;3200 $^b$</td>
<td>N/a</td>
<td>&gt;4900 $^b$</td>
<td>N/a</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>16.0±0.4 $^c$</td>
<td>36.0±4.9</td>
<td>0.4</td>
<td>204±37.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>87.2±4.2</td>
<td>139±28</td>
<td>0.6</td>
<td>458±2</td>
<td>0.2</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>193±30</td>
<td>52.3±11.9</td>
<td>3.7</td>
<td>490±81</td>
<td>0.4</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>2342±305 $^c$</td>
<td>298±70</td>
<td>7.9</td>
<td>~3000 $^b$</td>
<td>N/a</td>
</tr>
<tr>
<td>Cd$^{2+}$</td>
<td>7.3±1.1 $^c$</td>
<td>24.1±3.6</td>
<td>0.3</td>
<td>64.1±8.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

$^a$ standard deviations also listed; $^b$ estimated, saturation not observed; $^c$ from reference (10)

Table 2.3. Binding of MntR, MntR E99C, and MntR D8M proteins to the mntA recognition sequence. Dissociation constants ($K_d$) for modeling the fluorescence anisotropy data with a 1:1 binding isotherm. All values are based on an average of at least two independent experiments.

<table>
<thead>
<tr>
<th>Metal Ion (1 mM)</th>
<th>MntR $K_d$ (nM) $^a$</th>
<th>MntR E99C $K_d$ (nM) $^a$</th>
<th>$K_{d-w.t.}/K_{d-E99C}$</th>
<th>MntR D8M $K_d$ (nM) $^a$</th>
<th>$K_{d-w.t.}/K_{d-D8M}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo</td>
<td>&gt;3500 $^b$</td>
<td>&gt;3500 $^b$</td>
<td>N/a</td>
<td>&gt;3500 $^b$</td>
<td>N/a</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>30.4±1.3</td>
<td>25.8±6.4</td>
<td>1.2</td>
<td>93.0±23.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>79.8±20.8</td>
<td>103±12</td>
<td>0.8</td>
<td>267±26</td>
<td>0.3</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>81.5±13.8</td>
<td>26.4±5.1</td>
<td>3.1</td>
<td>241±27</td>
<td>0.3</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>507±38</td>
<td>94.8±9.4</td>
<td>5.4</td>
<td>1122±151</td>
<td>0.4</td>
</tr>
<tr>
<td>Cd$^{2+}$</td>
<td>24.3±4.6</td>
<td>19.8±6.0</td>
<td>1.2</td>
<td>37.7±4.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

$^a$ standard deviations also listed; $^b$ estimated, saturation not observed
Figure 2.9. Representative fluorescence anisotropy data for MntR E99C (left) and MntR D8M (right) binding to the oligonucleotide dsmntH26. Binding in the presence of 1.0 mM Mn$^{2+}$ (open circles) and Ni$^{2+}$ (filled circles) are shown. Lines through each set of points represent a fit of the data to a 1:1 oligonucleotide/dimer binding isotherm. [dsmntH26] = 10 nM. Buffer = 20 mM HEPES pH 7.2 @ 4 °C, 500 mM NaCl, 5% (v/v) glycerol. $T = 25$ °C.

Experiments with ANS fluorescence showed that the MntR proteins underwent differing degrees of structural organization based on the concentration and nature of added metal ion. For example, at 0.1 mM Cd$^{2+}$ MntR E99C appears completely folded, while the same concentration of Mn$^{2+}$ appears insufficient to induce significant folding (Figure 2.8). Nevertheless, at high concentrations of either metal (1.0 mM), MntR E99C showed strong activation for DNA-binding with nearly equal affinity for the cognate sequences mntH and mntA. These observations suggested that the differences observed in the ANS experiments are likely due to differences in metal binding affinity. To further examine this hypothesis, DNA-binding fluorescence anisotropy experiments with MntR E99C and dsmntH26 were performed at reduced metal concentrations. Binding of dsmntH26 by MntR E99C with 0.1 or 0.01 mM of Cd$^{2+}$ ($K_d \approx 19$ and 26 nM,
respectively) was essentially identical to that found in the presence of 1.0 mM Cd$^{2+}$ (Figure 2.10). In contrast, the DNA-binding ability of MntR E99C with 0.1 mM Mn$^{2+}$ was significantly reduced with a $K_d$ value of $\sim$209 nM (Figure 2.10). This suggests that MntR E99C binds Cd$^{2+}$ with higher affinity than Mn$^{2+}$, even though at high concentrations both metals are comparable activators for DNA binding.

**Figure 2.10.** Representative fluorescence anisotropy data for MntR E99C binding to the oligonucleotide dsmntH26 in presence of Cd$^{2+}$ (left) and Mn$^{2+}$ (right). Binding in the presence of 1.0 mM M$^{2+}$ (open circles, solid line) and 0.1 mM M$^{2+}$ (filled circles, dashed line) and 0.01 mM M$^{2+}$ (×, dotted line, Cd$^{2+}$ only) are shown. Lines though each set of points represent a fit of the data to a 1:1 oligonucleotide/dimer binding isotherm. Data show that binding affinity for dsmntH26 is invariant within this range of Cd$^{2+}$ concentrations but drops when the concentration of Mn$^{2+}$ is reduced. [dsmntH26] = 10 nM. Buffer = 20 mM HEPES pH 7.2 @ 4 °C, 500 mM NaCl, 5% (v/v) glycerol. $T$ = 25 °C.
DISCUSSION

**Mechanism of MntR Activation**

Addition of activating metal ions to MntR does not measurably change the CD feature at 222 nm, suggesting that the largely \( \alpha \)-helical secondary structure of MntR does not require, nor is substantially altered by, metal binding. In contrast, the thermal stability of the protein, as evaluated by CD spectroscopy, is significantly enhanced by the presence of activating metal ions such as \( \text{Mn}^{2+} \) and \( \text{Cd}^{2+} \). Metal ions such as \( \text{Mg}^{2+} \) and \( \text{Ca}^{2+} \), which do not activate the protein for DNA binding, have no effect on protein stability. These CD experiments indicate that although the secondary structure of MntR is not metal ion dependent, metal binding greatly stabilizes these structural features. Similar observations have been made with the nickel(II)-responsive metalloregulator NikR, where certain transition metal ions are found to stabilize one domain of NikR, with \( \text{Ni}^{2+} \) showing a selective ability to stabilize both domains of the protein (24).

ANS fluorescence experiments with MntR clearly demonstrate that metal binding results in a considerable reduction of the available hydrophobic surface area and generation of a more compact protein fold. This observation is consistent with ANS experiments performed on an iron(II)-responsive homologue DtxR (16). The ANS fluorescence data indicate that apo MntR undergoes a significant structural rearrangement upon metal coordination that leads to allosteric activation of the protein for DNA binding; however, while ANS binding allows us to stipulate that the fluorescence quenching occurs due to a decrease of available hydrophobic surface that accompanies protein reorganization, the pathway and origin of this rearrangement...
cannot be unambiguously determined. One possibility is that this organization stems from a rearrangement of the quaternary structure of MntR. Although apo MntR is a stable homodimer (10), the observed reorganization may occur by a reorientation of the two monomer subunits relative to one another, optimizing the orientation and spacing of the helix-turn-helix motifs for DNA binding and reducing the amount of exposed hydrophobic surface in the process. This type of quaternary structural compaction has been observed in SmtB, a zinc-responsive metalloregulatory protein of the ArsR family (27, 28). SmtB is a negatively regulated repressor of transcription from cyanobacteria that has a high affinity for its cognate DNA sequence in the apo form and a reduced affinity upon metal binding. Crystallographic and NMR solution studies show that zinc(II) binding to SmtB causes a quaternary structural contraction that places the DNA recognition elements in a sub-optimal conformation (28). A second possibility is that MntR undergoes an internal tertiary structural rearrangement that results in a loss of exposed hydrophobic surface area that leads to an optimized MntR structure for DNA binding. Such a tertiary structural rearrangement for MntR may originate from a molten globule-like state with defined secondary structural elements, but a less organized tertiary structure (29). This scenario has been proposed for DtxR, where the protein was observed to be in a molten globule state prior to metal binding (16).

When viewed in the context of the available structural data on MntR (11, 17) and the observation that apo MntR exists as a stable homodimer (10), we favor a model that invokes a change in tertiary conformation of MntR that acts as an allosteric trigger for DNA binding. Such a model is consistent with the observation that the metal binding site in MntR recruits ligands from every major structural region of the protein:
Asp8 and Glu11 from the helix-turn-helix DNA binding domain (Figure 2.1, red), Glu99, Glu102, and His103 from the dimerization domain (Figure 2.1, blue), and His77 from the long α-helix that links the dimerization and DNA binding domains (Figure 2.1, green). It is feasible that binding of a metal ion to this active site leads to a widespread structural rearrangement of the protein secondary structures to form a stable, activated repressor complex. This is also consistent with the generally defective behavior of MntR D8M, which alters and lacks several of these key binding interactions (Figure 2.2), rendering it incapable of completely organizing the protein tertiary structure upon binding of a transition metal ion (vide infra). Whether this tertiary structure reorganization originates from a molten globule state or from a relatively well-structured MntR conformation will best be resolved by NMR solution studies.

CD thermal denaturation and ANS fluorescence experiments suggest that the mechanism of repressor activation is related to substantial ordering and stabilization of the protein structure at the tertiary level. The significance of these findings is illustrated by comparison to other metalloregulatory proteins such as DtxR and CzrA. X-ray diffraction studies of both the apo and holo forms of DtxR show minimal variance in structure (18), making it difficult to account for functional differences between the two forms or gain insight into the metal-mediated activation of the protein. X-ray structural characterization of the metalloregulatory protein CzrA also shows nominal changes in overall structure between the apo and holo forms (28), again limiting functional and mechanistic insight. An improved understanding of metal activation for both DtxR and CzrA was ultimately obtained through solution studies. In the case of DtxR, metal binding was found to organize the tertiary structure into a favorable configuration in a
disorder-to-order transition (16). For CzrA metal complexation resulted in reduced structural dynamics that served to “freeze out” a configuration required for DNA binding (28). The studies on DtxR and CzrA highlight the importance of our experiments with MntR, as the significant structural changes found for MntR in solution have not been previously investigated and appear to be an important part of the metal-mediated mechanism for DNA binding. Further investigation into solution dynamics of MntR using NMR spectroscopy will provide a more complete understanding into the mechanism of activation.

DNA Binding Selectivity

Apo MntR has a low affinity for both the \textit{mntH} and \textit{mntA} cognate sequences, as judged by fluorescence anisotropy measurements. Binding of MntR is dependent on the presence of transition metal ions with the general trend for DNA-binding ability (at 1.0 mM metal ion) following $\text{Cd}^{2+} \approx \text{Mn}^{2+} > \text{Fe}^{2+} \approx \text{Co}^{2+} >> \text{Ni}^{2+} >>$ apo. These findings are consistent with an earlier hypothesis that MntR acts as a manganese(II)-regulated repressor of both the \textit{mntH} and \textit{mntABCD} operons, confirming previous studies using DNase I footprinting experiments (30). The binding studies presented here also show that MntR generally has comparable affinities for the \textit{mntH} and \textit{mntA} sequences when activated by transition metal ions. The two new metal ions investigated in this study, Fe$^{2+}$ and Co$^{2+}$, were found to be intermediate activators indicating that there is a gradation of levels to which MntR can be activated to bind DNA. Overall, the metal-selective response of MntR appears somewhat more stringent with \textit{mntH}, as activation with excess Mn$^{2+}$ is $\sim$150-fold more effective than Ni$^{2+}$, while excess Mn$^{2+}$ activates
MntR only ~17-fold more strongly than Ni^{2+} for mntA. The presence of excess metal ion in the fluorescence anisotropy experiments limits unambiguous interpretation of the data and makes the origin of metal-selective activation unclear; however, evidence suggests (Chapter 3 of this thesis) that differences in metal binding affinities alone (9) are insufficient to explain the metal specific response of MntR.

As found in earlier reports, MntR is best activated by two very disparate metal ions, Mn^{2+} and Cd^{2+} (8, 10). Why MntR responds so strongly to these metal ions and why the Cd^{2+} response may be biologically relevant is a topic of particular interest. Interestingly, DNA-binding experiments performed at reduced metal concentrations provide some likely insight into the metal binding affinities of MntR, which may serve to address the latter question. In the presence of 1.0 mM Mn^{2+} or Cd^{2+}, MntR E99C binds dsmntH26 with comparable affinity. In contrast, at 0.1 mM Mn^{2+} affinity drops ~10-fold, but remains essentially unchanged with Cd^{2+} even at concentrations as low as 0.01 mM metal. This finding supports the hypothesis that metal ion affinity does play a role in the metal-selective response of MntR and is discussed in further detail in Chapter 3 of this thesis (9). A greater affinity for Cd^{2+} may play an important physiological role for MntR. The proton-dependent Mn^{2+} transporter MntH has been shown to facilitate Cd^{2+} uptake in B. subtilis (8). If Cd^{2+} is assumed to be a non-essential, and likely toxic metal ion for this organism, then it would be important that MntR be able to respond to small amounts of Cd^{2+} inadvertently taken up through the MntH transporter in a cadmium(II)-contaminated environment. Furthermore, the sensitivity of MntR to Cd^{2+} may also explain why B. subtilis has several pathways for Mn^{2+} uptake (8). In the presence of small amounts of Cd^{2+}, the MntH (and MntABCD)
transport system would be repressed to prevent transport of the toxic metal, but alternative pathways might still allow for essential quantities of Mn\(^{2+}\) to be taken into the cell.

**Active Site Mutants**

Recent in vivo studies have been helpful in clarifying the role and origin of the metal-selective response by MntR and DtxR. Although the normally iron(II)-responsive DtxR could be changed to a manganese(II)-selective mutant, mutants of MntR were always found to be manganese(II)-responsive (9). In reporter genes assays, Mn\(^{2+}\) remained the preferred activator for both MntR E99C and MntR D8M; however, MntR E99C showed no gene repression in response to Fe\(^{2+}\), while MntR D8M could be activated by Fe\(^{2+}\), albeit at higher concentrations than required with Mn\(^{2+}\) (9). The fluorescence anisotropy binding experiments to dsmntH26 described here with MntR E99C and MntR D8M are qualitatively consistent with the in vivo findings. Relative binding affinities for both cognate sequences in the presence of Mn\(^{2+}\) and Fe\(^{2+}\) remain essentially unchanged between MntR and MntR E99C. In contrast, MntR D8M shows a marked loss of selectivity for Mn\(^{2+}\), suggesting that a loss of Mn\(^{2+}\) sensitivity and not a gain of Fe\(^{2+}\) function is responsible for the in vivo responsiveness of this mutant to both Mn\(^{2+}\) and Fe\(^{2+}\) in *B. subtilis*.

The biophysical studies on MntR D8M presented here show that this mutation is highly detrimental to the function of this transcription factor. Thermal denaturation studies demonstrate that apo MntR D8M has a similar denaturation transition temperature to wild type; however, MntR D8M is far less resistant to thermal
denaturation in the presence of Mn$^{2+}$, Ni$^{2+}$, and Cd$^{2+}$. Experiments with the fluorescent probe ANS support the thermal denaturation results, as Mn$^{2+}$, Ni$^{2+}$, and Cd$^{2+}$ induce a smaller decrease in ANS fluorescence in MntR D8M when compared to wild type MntR. ANS data indicate that only Co$^{2+}$ retains a good ability to organize MntR D8M (Figure 2.8), and this is consistent with the thermal denaturation curves that show Co$^{2+}$ stabilization of MntR D8M is the least perturbed when compared to wild type (Table 2.1). Giedroc and co-workers have discussed active site mutations of metalloregulators in the context of SmtB/ArsR family of proteins, and have provided three possible explanations for the defective activity of mutant proteins (27): a) the mutant has lost the ability to bind metal ions, b) the mutant protein binds metal ions, but with substantially reduced thermodynamic affinity, or c) the mutant binds metal ions with high affinity, but in a non-native coordination geometry that results in a non-functional protein. The binuclear nature of the native MntR binding site somewhat complicates this classification scheme, but clearly MntR D8M has not completely lost the ability to bind metal ions as shown by the experiments described herein, as well as the crystal structure of holo MntR D8M, where one of the two metal-binding sites was found to be occupied (11). Under the experimental conditions employed here, which utilize a large excess of metal ion, it is not possible to unambiguously determine which of the remaining possibilities is most relevant to MntR D8M. The crystal structure of MntR D8M shows a single manganese(II) ion bound in a somewhat modified coordination environment (Figure 2.2), but whether this site retains a high affinity for metal binding has not yet been determined.
Overall, our findings are consistent with the structure of holo MntR D8M (Figure 2.2), which shows a disruption of the binuclear metal binding site (11). DNA binding studies with MntR D8M show that this protein is functionally deficient, showing only weak activation upon binding of transition metal ions. The results obtained here and the reported X-ray structure of MntR D8M suggest that occupation of metal binding site ‘C’ may be essential to proper function of the protein (11). The deficiencies in MntR D8M behavior observed here do lend support to the hypothesis that the binuclear site is an important factor in activating MntR, at least in the case of Mn$^{2+}$. While at present it is unclear exactly which aspects of the metal site in MntR are required for gene repression, the binuclear nature of the active site makes it unique among the DtxR family of metalloregulatory proteins that have been structurally characterized to date (11-15). Indeed, several metalloregulatory proteins that function as positively regulated repressors of transcription are known to employ more than one metal ion per monomer, but do so in two distinct metals sites. These sites can be broadly categorized into two groups, a high affinity ‘structural’ site and a lower affinity ‘sensory’ or ‘allosteric’ site. For example, in NikR a high affinity site activates the protein to bind DNA when occupied by a variety of metal ions; however, a second low affinity site in NikR is selective for the nickel(II) ion (31). In the metalloregulatory proteins Fur and Zur, a high affinity site binds a zinc(II) ion that is proposed to play a largely structural role and a lower affinity site is proposed to be the selective sensory site for iron(II) and zinc(II), respectively (32-34). In DtxR, the ancillary site (Figure 2.2, site A) is proposed to be a structural site, while the primary site (Figure 2.2, site C) is required for DNA binding activity (3). In contrast, MntR may have fused the
functions of both a quintessential structural and sensory site by having a single
binuclear site. More extensive mutagenesis, structural, and biophysical studies will be
necessary to elucidate all of the structural dynamics and metal site requirements for
MntR activation.
MATERIALS AND METHODS

General

All buffers were prepared using water purified through a Labconoco Water Pro Plus purification system. Buffers were degassed and sterilized by passing them through 0.22 μm filters. All biochemical reagents were obtained from commercial suppliers and were used as provided. MnCl₂·4H₂O, FeCl₂·4H₂O, CoCl₂·H₂O, CdCl₂·H₂O, and NiCl₂·6H₂O (99.99+% ) were obtained from Aldrich. All protein chromatography was performed at 4 °C on an ÄKTA Prime biomolecule purification system (Amersham Pharmacia Biotech) using both inline UV and conductance detection. Matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry was performed by HT Labs (San Diego, CA) or at the core facility at the University of California, San Diego. The metal content/purity of all preparations of proteins, ultrapure water, buffers, and metal titrant solutions was determined as previously described (10) using a Perkin-Elmer Optima 3000 DV inductively coupled plasma optical emission spectrometer (ICP-OES) located at the Analytical Facility at the Scripps Institute of Oceanography. All fluorescence intensity and anisotropy experiments were performed on a Perkin-Elmer Luminescence Spectrometer LS 55 using a thermally-jacketed cell holder that was maintained at 25 °C. Size exclusion chromatography was performed on a calibrated HiLoad 16/60 Superdex 75 Size Exclusion column (Amersham Pharmacia Biotech) as previously described (10). Analytical sedimentation equilibrium ultracentrifugation experiments were performed as previously described (10) on a Beckman XL-1 Analytical Ultracentrifuge located at the Biophysics Instrumentation
Facility in the Department of Chemistry and Biochemistry at the University of California, San Diego. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, Iowa).

**Expression and Purification of MntR**

BL21-CodonPlus(DE3)-RIL *E. coli* cells (Stratagene) transformed with pET17b plasmid containing the wild type MntR (8) gene or the mutant genes MntR E99C or MntR D8M (11) were expressed and purified as previously described (10) with minor modifications. Buffers were as follows: lysis buffer consisted of 50 mM Tris, pH 8.0 @4 °C, 200 mM NaCl, 5 mM EDTA, 5% (v/v) glycerol, 5 mM β-mercaptoethanol; cation exchange column buffer consisted of 50 mM HEPES, pH 7.0 @4 °C, 150 mM NaCl, 5% (v/v) glycerol; storage buffer consisted of 20 mM HEPES, pH 7.2 @4 °C, 200 mM NaCl, 5% (v/v) glycerol. MntR E99C and MntR D8M storage buffer also included 100 μM β-mercaptoethanol, and MntR D8M was stored at a higher salt concentration (300 mM NaCl) to prevent protein precipitation. The proteins were quantified using calculated extinction coefficients of 18910 M⁻¹ cm⁻¹ for wild type and MntR D8M, and 19035 M⁻¹ cm⁻¹ for MntR E99C (20).

**Determination of Free Cysteines**

MntR E99C was dialyzed for 2 × 4 h against deoxygenated storage buffer to remove β-mercaptoethanol contained in the original storage buffer for this mutant (vide supra). The dialysis was performed inside a glovebox containing a nitrogen atmosphere by using 500 μL capacity cassettes (Slide-A-Lyzer, 3.5 kDa MWCO, Pierce).
portion of the dialyzed protein sample (200 μL) was mixed with 200 μL of 7 M urea/2.5 M EDTA to give reaction mixtures containing 10-40 μM protein. To this mixture, 25 μL of 2.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) solution (prepared in storage buffer) was added and the mixture was agitated for 30 min. The reaction mixtures were taken out of the glovebox and analyzed spectrophotometrically by monitoring the absorbance at 412 nm using the reported extinction coefficient of 13,600 M⁻¹ cm⁻¹ (21). The absorbance value at 412 nm was corrected by subtracting the spectrum of a buffer solution containing same concentration of DTNB, but without any protein. MntR E99C preparations analyzed in this manner were found to contain 0.88±0.12 free thiols per monomer.

**Circular Dichroism Spectroscopy**

Circular dichroism (CD) measurements were made on an AVIV Circular Dichroism Model 202 Spectrometer using a 2.0 mm quartz cuvette. The cuvettes were washed with soapy water, 1.0 M HCl, and copious amounts of ultrapure water followed by an ethanol rinse and air drying to ensure the cells were clean and free of metal contaminants. CD spectra (190 – 260 nm) were collected for each sample with a 0.5 nm step and a 4.0 s averaging time. Samples were supplemented with metal ions as desired from stock solutions (~5.0 mM) in storage buffer to a concentration of 1.0 mM. For thermal denaturation studies identical solution conditions were applied. The thermal denaturation profile for apo MntR was found to be identical in sodium phosphate or HEPES (storage) buffer (Figure 2.3); however, under our experimental conditions the addition of metal ions to phosphate buffered solutions resulted in precipitation of metal
phosphates. Therefore, the thermal denaturation experiments were performed in storage buffer to minimize complications with metal binding experiments.

Samples containing \( \sim 8 \mu M \) protein were heated from 50 – 100 \( ^\circ C \) at a rate of 1.0 \( ^\circ C/\text{min} \) and ellipticity was monitored at 222 nm; melting temperatures \( (T_M) \) were determined as previously described (22). Select runs initiated at a lower starting temperature (25 \( ^\circ C \)) showed no difference in the denaturation curves. \( T_M \) values were calculated with Equation 1 and solving for the temperature \( (T) \) at which \( \Delta G = 0 \):

\[
\Delta G = -RT \ln \left( \frac{I - I_F}{I_U - I} \right)
\] (2.1)

where \( I \) is ellipticity at 222 nm at temperature \( T \), \( I_F \) is the initial (folded) ellipticity, and \( I_U \) is the final (unfolded) ellipticity. Initial/folded intensity \( (I_F) \) and final/unfolded intensity \( (I_U) \) were taken from the first and last points of each individual denaturation curve. Due to lack of reversibility (non-equilibrium conditions) meaningful thermodynamic parameters could not be obtained. Data are presented as fractional change in ellipticity (FCE) at 222 nm by using the Equation 2:

\[
FCE = \frac{(I_F - I)}{(I_F - I_U)}
\] (2.2)

**Fluorescence Emission with ANS**

500 \( \mu M \) 8-Anilino-1-naphthalenesulfonic acid (ANS) and 5 \( \mu M \) protein sample in storage buffer (initial solution volume \( \sim 380 \mu L \)) were placed in a microcuvette (800 \( \mu L \)). Fluorescence spectra were collected using an excitation wavelength of 403 nm (slit width 15 nm) and an emission wavelength of 380 – 600 nm (slit width 20 nm). Data were an average of five scans. In the presence of protein, the emission maximum
for ANS was found to blue shift 5 – 10 nm from the protein free value of 515 nm; therefore, comparative analysis of spectra was performed at 507 nm. Metal ions were added from stock solutions in storage buffer, to concentrations of approximately 0.1, 0.5, and 1.0 mM. Control experiments showed that under identical solution conditions, but in the absence of protein, no change in the fluorescence spectra of 500 μM ANS was observed in the presence of 1.0 mM Mg2+, Ca2+, Mn2+, Co2+, Ni2+, or Cd2+.

**Fluorescence Anisotropy Titrations**

The same procedure for preparing the double-stranded probe dsmntH26 was applied for both dsmntH26 and dsmntA26 used in this study (10). The modified oligonucleotide ssmntA26 (5'-6F-GATAATTTTGCGGAACTTC-3', where the fluorescein dye is indicated by 6F = 6-carboxyfluorescein) and the complementary oligonucleotide (5'-GAAAGTTTCCCTCAGCAAATTAC-3') were used to generate the double-stranded oligonucleotide dsmntA26 containing the desired recognition sequence. The sequence of the labeled strand of dsmntH26 is 5'-6F-GAAATTTTGCTTAAGGAAACTCTC-3'. Fluorescence anisotropy experiments were performed as previously described with some modifications (10). Measurements were collected with an excitation wavelength of 492 nm (slit width 15 nm), an emission wavelength of 520 nm (slit width 20 nm), and a 1.0 s integration time. The g-factor for all of the experiments was 1.15±0.08. For titrations in the presence of 1.0 mM Fe2+ the experiments were performed by using an anaerobic cell assembled inside a glove box with a nitrogen atmosphere. The \( r_{obs} \) versus [MntR]total data were fit to a 1:1 binding
isothesis model (non-dissociable dimer) \((10)\) by using least-squares regression analysis software (Synergy KaleidaGraph).

The text of chapter 2, in full, is a reprint of the material as it appears in the journal Biochemistry 2005, vol. 44(9):3380-9 “Metal-Induced Structural Organization and Stabilization of the Metalloregulatory Protein MntR” . The dissertation author was the primary researcher and/or author and the co-authors (Davis TC, Helmann JD, Cohen SM.) listed in this publication contributed to or supervised the research which forms the basis for this chapter. The authors would like to thank Dr. Emmanuel Guedon (Helmann Lab, Cornell University) for providing the protein vectors, Prof. Elizabeth Komives (U.C. San Diego) for assistance with use of the analytical ultracentrifuge and MALDI-TOF mass spectrometer, Prof. Patricia A. Jennings for assistance with the CD measurements and many helpful discussions, and Dr. Kevin Walda, Dr. Annette Deyhle, and Christopher Mahn (Scripps Institute of Oceanography) for access and assistance with the ICP-OES.
REFERENCES


Chapter 3.

Metal Binding Studies and EPR Spectroscopy of the Manganese Transport Regulator MntR
ABSTRACT

Prior biophysical studies from our lab have focused on the metal-mediated DNA binding of MntR, as well as metal stabilization of the MntR structure, but only limited data on the metal-binding affinities for MntR are available. Herein, the metal-binding affinities of MntR were determined by using EPR spectroscopy, as well as competition experiments with the fluorimetric dyes Fura-2 and Mag-fura-2. MntR was not capable of competing with Fura-2 for the binding of transition metal ions. Therefore, the metal-binding affinities and stoichiometries of Mag-fura-2 for Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$ were determined, and utilized in MntR/Mag-fura-2 competition experiments. The measured $K_d$ values for MntR metal binding are comparable to those reported for DtxR and AntR metal binding, and generally follow the Irving-Williams series. Direct detection of the binuclear Mn$^{2+}$ site in MntR with EPR spectroscopy is presented and the exchange interaction was determined, $J = -0.2$ cm$^{-1}$. This value is lower in magnitude than most known binuclear Mn$^{2+}$ sites in proteins and synthetic complexes, and is consistent with a binuclear Mn$^{2+}$ site with a longer Mn⋯Mn distance (4.4 Å) observed in some of the available crystal structures. MntR is found to have a surprisingly low binding affinity (~160 μM) for its cognate metal ion Mn$^{2+}$. Moreover, the results of DNA binding studies in the presence of limiting metal ion concentrations were found to be consistent with the measured metal binding constants. The metal binding affinities of MntR reported here help to elucidate the regulatory mechanism of this metal-dependent transcription factor.
INTRODUCTION

To date, a large number of metalloregulatory proteins have been identified that respond to a variety of metal ions (1-3). The subject of how metal binding is translated into an ability to control transcription via a metalloregulator has become a prominent subject of investigation (4-10). Indeed, many metalloregulators are reported to bind several metal ions in vitro, while only eliciting a specific transcriptional response when they are bound to the cognate metal in vivo (5-7, 9). This observation naturally leads to the question, how does a metalloregulator selectively respond to its cognate metal ion as opposed to other available metal ion activators? The ability of a metalloregulator to respond selectively to a metal ion may depend on several factors, including: the availability of the requisite metal ion, the binding affinity for the metal ion, the charge on the metal ion, and the coordination geometry/number assumed by the metal ion upon binding. Determining which of these factors are most important for a given metalloregulator is essential for gaining a better understanding of how these proteins elicit transcriptional control.

In case of MntR, it has been shown that like other members of the DtxR family, MntR requires the binding of two metal ions per protein monomer and utilizes a helix-turn-helix DNA-binding motif (11, 12). The two metal ions in MntR form a bridged, binuclear metal active site (M⋯M distances ranging from ~3.3 to 4.4 Å), which is distinct from the two mononuclear sites (M⋯M distance ~9 Å) found in DtxR (12-14). Interestingly, unlike DtxR, which is an iron responsive repressor (15), MntR is responsive to manganese and cadmium in vivo (16). This observation raises the issue of
how metal selectivity is achieved by MntR and is the focus of this chapter. We have already investigated the effect of metal-site mutations (MntR D8M and MntR E99C) in MntR on the structure of this protein and its ability to bind DNA (see chapter 2) and herein further investigate the effect of these mutations on the metal binding affinities of MntR.

Structural studies on MntR show that, with the exception of the metal site, the overall protein structure is the same for various metal isoforms (12, 14) (Figure 3.1).

![Figure 3.1](image)

**Figure 3.1.** Overlaid crystal structures of MntR. Proteins are depicted as cartoons, metal atoms are shown as spheres. Overlay shows Mn$^{2+}$-MntR over Cd$^{2+}$-MntR (left, RMS 0.265 Å), Mn$^{2+}$-MntR over Zn$^{2+}$-MntR (center, RMS 0.632 Å) and Zn$^{2+}$-MntR over Mn$^{2+}$-MntR-D8M (right, RMS 0.718 Å). The figure illustrates that tertiary and quaternary structure of MntR is quite similar between the isoforms. MntR isoforms are colored blue (Mn$^{2+}$-MntR, PDB ID: 2F5F), red (Cd$^{2+}$-MntR, PDB ID: 2EV0), green (Zn$^{2+}$-MntR PDB ID: 2EV6) and faint blue (Mn$^{2+}$-MntR-D8M, PDB ID: 1ON2)

As for the metal-binding site, three different arrangements have been observed for wild type MntR: (1) a binuclear AB conformer with a 3.3 Å M···M distance, (2) a binuclear AC conformer with a 4.4 Å M···M distance, and (3) a mononuclear conformer with only the A site occupied (Figure 3.2). The AB and AC binuclear clusters utilize the same protein ligands, but the binding mode and side chain orientations in the two
forms are different. The AC form of MntR has been observed upon reconstitution with Ca$^{2+}$, Mn$^{2+}$, and Cd$^{2+}$, while the AB form has only been observed with Mn$^{2+}$ \((12, 14)\).

![Figure 3.2. Physiologically relevant metal sites of MntR. Variants of binuclear metal site AC are displayed on the left (Mn$^{2+}$-MntR, top and Cd$^{2+}$-MntR, bottom) while variants of mononuclear metal site A are displayed on the right (octahedral Mn$^{2+}$-MntR-D8M, top and tetrahedral Zn$^{2+}$-MntR, bottom). Metal atom color-coding is adopted from Figure 3.1. H$_2$O molecules are displayed as red spheres, nitrogen, oxygen and sulfur atoms are colored in blue, red and yellow; metal-ligand bonds are displayed as dashed black lines.](image)

With Mn$^{2+}$, formation of the AB versus AC form is dependent upon conditions such as temperature and pH; from these studies the AC form has been proposed as the more physiologically relevant conformer \((14)\). The mononuclear A only site form has been
observed with Co\textsuperscript{2+} (A. Glasfeld personal communication) and Zn\textsuperscript{2+} (14). Based on the observation that the A site is conserved in all three structure types, it has been suggested that the A site serves as an ‘activation filter’. Further, considering that Mn\textsuperscript{2+} and Cd\textsuperscript{2+} are strong activators for DNA binding of MntR while Zn\textsuperscript{2+} is poor, the formation of a binuclear site is essential for full activation of the protein (14). In particular, it was hypothesized that the coordination observed in Zn\textsuperscript{2+}-MntR was suboptimal and did not facilitate binding of a second metal ion, and this second binding event is proposed to be required for complete activation of MntR. This hypothesis is consistent with the metal-activation profile of MntR, which shows that the formation of a binuclear transition metal site results in strong activation for DNA binding, while formation of a mononuclear metal site results in either diminished or abolished DNA-binding activity (11, 17).

Prior studies on MntR have focused on the metal activation profile (see chapter 2), which showed that in vitro an excess of Mn\textsuperscript{2+} or Cd\textsuperscript{2+} ions (1.0 mM, >100-fold concentration relative to protein present) results in tight DNA binding (11). Other metal ions, such as Fe\textsuperscript{2+} and Co\textsuperscript{2+}, were found to elicit moderate operator binding as well (17). Previous studies suggest that binding of these activating metal ions to MntR result in a change in protein tertiary structure, while the secondary structure remains largely unaltered (11, 17). Despite the insight gained from these investigations, the studies were typically performed in the presence of excess metal ions (>100-fold excess), complicating the interpretation and physiological relevance of these findings. To better understand these results, detailed knowledge of the metal binding of MntR is required. Two recent reports have examined metal binding in MntR and related
homologs. In the first study, several new crystal structures, with Ca$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$, along with calorimetry data on metal binding was reported for MntR (14). In the second study, a close homologue of MntR, AntR from *B. anthracis*, was probed by EPR spectroscopy to examine metal binding and oligomerization behavior (18). Herein, we present the metal-binding affinities of w.t. MntR, MntR D8M and MntR E99C for several transition metal ions in an attempt to complement earlier biophysical studies and to compare our findings to the recent reports on MntR and AntR. By measuring the metal-binding affinities of MntR, and relating them to the reported DNA-binding activation profile, we can refine our interpretation of these prior findings, as well as determine whether a high metal binding affinity is essential for DNA binding activation of MntR. The results presented in this chapter also point to a possible explanation for the Mn$^{2+}$/Cd$^{2+}$ dual responsiveness of MntR.
RESULTS

ANS Experiments

The fluorescence of the ANS dye increases upon binding to hydrophobic surfaces; prior studies with ANS and MntR were used to monitor changes in protein tertiary/quaternary structure at several metal ion concentrations (17). The fluorescence of ANS in buffered solution increases upon addition of MntR, and then successively decreases upon addition of increasing amounts of activating metal ions (e.g. Mn\textsuperscript{2+}), indicating a change in protein structure and a burying of hydrophobic surfaces within MntR. Using this decrease in fluorescence as a probe, much like the quenching of tryptophan fluorescence, ANS can be used as an exogenous reporter (7, 9). Titration curves of fluorescence emission intensity versus metal ion concentration were obtained for MntR with Mn\textsuperscript{2+}, Co\textsuperscript{2+}, and Cd\textsuperscript{2+}. Under these conditions, MntR binds Mn\textsuperscript{2+}, Co\textsuperscript{2+}, and Cd\textsuperscript{2+} with \(K_d\) values of 92, 13, and 0.5 \(\mu\text{M}\), respectively (Figure 3.3). This initial study revealed that the cognate metal ion, Mn\textsuperscript{2+}, is bound with the weakest affinity relative to other metal ions that activate MntR for DNA binding. Due to several limitations of using ANS as a probe for metal ion binding (vide infra), a more quantitative method for determining the metal binding affinities of MntR was sought. The fluorimetric dyes Fura-2 and Mag-fura-2 (Figure 3.4) were used in competition experiments with MntR to obtain quantitative data.
Figure 3.3. Titration of Mn$^{2+}$ (○), Co$^{2+}$ (■), or Cd$^{2+}$ (×) into a solution of MntR (2 μM) and ANS (200 μM) in protein storage buffer. The ANS fluorescence at 507 nm is quenched as MntR binds to the metal ions. Fits of the data using a 1:1 binding isotherm are shown as solid lines. $T= 25$ °C.

Figure 3.4. Structures of Fura-2 (left) and Mag-fura-2 (right). Both compounds are obtained as the potassium salts.
Protein Competition with Fura-2

Originally designed as a molecular sensor for Ca\textsuperscript{2+}, the binding affinity of Fura-2 for several transition metal ions has been determined (19-22), and this fluorophore has been employed as a tool to study metal binding with the metalloregulatory protein NikR (6). Dyes such as Fura-2 are useful in this capacity as both their absorption and fluorescence spectra change upon binding to metal ions, providing a direct spectroscopic handle for evaluating metal binding affinities when in competition with metalloproteins. Competition experiments between Fura-2 and MntR were carried out by preincubating 5 μM dye and 50 μM MntR in protein storage buffer, after which 5 μM of the metal ion of interest (Mn\textsuperscript{2+}, Co\textsuperscript{2+}, Cd\textsuperscript{2+}) was added to the solution and absorption spectra were recorded overnight at 20 min intervals. In all experiments the spectrum of dye-metal complex remained unperturbed (data not shown), implying that MntR was incapable of competing with the dye for metal binding. Because MntR was present in a 10-fold excess over Fura-2, the affinity of MntR for these metal ions must be at least an order of magnitude weaker than that of Fura-2. This places the lower limit on the $K_d$ values of MntR at 30 nM for Mn\textsuperscript{2+}, 90 nM for Co\textsuperscript{2+}, and 10 pM for Cd\textsuperscript{2+}. It was concluded that MntR was unable to compete with Fura-2 for these metal ions; therefore, a fluorescent dye with weaker metal ion affinities was sought.

Metal-Binding Affinity/Stoichiometry of Mag-fura-2

Mag-fura-2 (23), a dye structurally similar to Fura-2 (Figure 3.4) but possessing a weaker Ca\textsuperscript{2+} affinity was investigated as an alternative to Fura-2. It was assumed that the weaker Ca\textsuperscript{2+} affinity of Mag-fura-2 relative to Fura-2 would also reflect a decrease
in transition metal ion affinities for Mag-fura-2. Unlike Fura-2, for which a number of metal binding affinities have been reported (19-22), the affinity of Mag-fura-2 has only been reported for the transition metal ion Zn$^{2+}$ (24). Therefore, we determined both the affinity and stoichiometry of Mag-fura-2 for Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$ in order to perform our studies with MntR.

Similar to Fura-2, Mag-fura-2 metal binding is accompanied by changes in its absorbance and fluorescence spectra. In particular, complexation of Mag-fura-2 with the closed-shell ions Ca$^{2+}$, Zn$^{2+}$, or Cd$^{2+}$ results in loss of fluorescent excitation at ~369 nm (indicative of apo-Mag-fura-2, $\lambda_{em}$=505 nm) with subsequent generation of a excitation band centered at ~330 nm (indicative of the dye-metal complex). Complexation of Mag-fura-2 with the paramagnetic ions Mn$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ results in quenching of the excitation band at ~369 nm. Using these spectral features, the dissociation constants for Mag-fura-2 were determined in both metal binding and protein storage buffer. A representative example of the spectra and model of the data for each type of competition titration (Co$^{2+}$ and Cd$^{2+}$) are shown in Figures 3.5 and 3.6. The $K_d$ values range from ~0.04 to ~1 μM (Table 3.1), with Zn$^{2+}$ < Ni$^{2+}$ ~ Cd$^{2+}$ < Mn$^{2+}$ ~ Co$^{2+}$. With the exception of Zn$^{2+}$ (24), the affinities of Mag-fura-2 for transition metal ions decrease by several orders of magnitude relative to Fura-2, consistent with that expected based on the difference in affinity of these two dyes for Ca$^{2+}$ (20, 23). The experiments performed in metal binding buffer are typical of the conditions often used for determining binding constants of fluorescent dyes and other small molecule ligands (20, 25). The $K_d$ values for Mag-fura-2 with these metal ions were also measured in protein storage buffer (Table 3.1), to ensure that the effects of pH and ionic
strength (relative to metal binding buffer) were minimal, and would not adversely impact the competition experiments with MntR. Finally, by using absorption spectroscopy and Job’s method (Figure 3.7) a 1:1 metal:Mag-fura-2 stoichiometry was confirmed for all of the metal ions examined.

**Table 3.1.** Dissociation constants \((K_d, \text{ with standard deviations})\) for Mag-fura-2 with several metal ions as determined in metal binding and protein storage buffer.

<table>
<thead>
<tr>
<th>Metal Binding Buffer (μM)</th>
<th>Protein Storage Buffer (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+}) 25(^a)</td>
<td>n/d</td>
</tr>
<tr>
<td>Mn(^{2+}) 0.97±0.17</td>
<td>0.89±0.03</td>
</tr>
<tr>
<td>Co(^{2+}) 0.93±0.04</td>
<td>0.92±0.06</td>
</tr>
<tr>
<td>Ni(^{2+}) 0.13±0.01</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td>Zn(^{2+}) 0.036±0.001(^d)</td>
<td>0.037±0.001</td>
</tr>
<tr>
<td>Cd(^{2+}) 0.10±0.01</td>
<td>0.16±0.01</td>
</tr>
</tbody>
</table>

\(^a\) Provided by the manufacturer, Invitrogen; \(^b\) determined by competition against Ca\(^{2+}\); \(^c\) determined by competition against Mn\(^{2+}\), Co\(^{2+}\), or Ni\(^{2+}\); \(^d\) 20 nM, I=0.15, T=37 °C, see ref. (24).
**Figure 3.5.** Fluorescence titration of Co$^{2+}$ into a solution of 1 mM Ca$^{2+}$ and 0.5 μM Mag-fura-2 in metal binding buffer. Fluorescence excitation spectra (left) shows the initial spectrum as a heavy solid line and the final spectrum is shown as a heavy dashed line. The spectra represent the emission intensity at 505 nm (ordinate) as a function of excitation wavelength (abscissa). Fit of the normalized fluorescence excitation intensity at $\lambda_{ex}=330$ nm (right) as a function of Co$^{2+}$ concentration. $T = 25$ °C.

**Figure 3.6.** Fluorescence titration of Cd$^{2+}$ into a solution of 1 mM Co$^{2+}$ and 0.5 μM Mag-fura-2 in metal binding buffer. Fluorescence excitation spectra (left) shows the initial spectrum as a heavy solid line and the final spectrum is shown as a heavy dashed line. The spectra represent the emission intensity at 505 nm (ordinate) as a function of excitation wavelength (abscissa). Fit of the normalized fluorescence excitation intensity at $\lambda_{ex}=330$ nm (right) as a function of Cd$^{2+}$ concentration. $T = 25$ °C.
Protein Competition with Mag-fura-2

Mag-fura-2 was used in competition experiments against MntR. These competition experiments were performed in a manner consistent with others described in the literature (26), with the metal ions of interest titrated into a solution containing both Mag-fura-2 and MntR. The competition between MntR and Mag-fura-2 for the titrated metal ion was monitored by fluorescence spectroscopy and in one case also confirmed by absorption spectroscopy (Co$^{2+}$) (Figures 3.8 - 3.10). Fluorimetric titration of Mn$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ into a solution of Mag-fura-2 with an excess MntR results in the quenching of the Mag-fura-2 fluorescence excitation band at ~369 nm ($\lambda_{\text{em}}$=505). Figure 3.8 shows a representative example of a titration performed with Co$^{2+}$. Titration of Zn$^{2+}$ and Cd$^{2+}$ results in the disappearance of a band at ~369 nm and formation of a new band at ~330 nm in the fluorescence excitation spectrum, indicative of the metal-
Figure 3.10 shows a representative competition titration in the presence of Cd\(^{2+}\). The data from all competition titrations were analyzed using Dynafit (27, 28) with a model that incorporated the relevant equilibria (see Addendum following “Materials and Methods”); for Mn\(^{2+}\), Co\(^{2+}\), and Ni\(^{2+}\) experiments data were fit using the fluorescence excitation at 380 nm, while for Zn\(^{2+}\) and Cd\(^{2+}\) titrations the data were fit using the fluorescence excitation at 330 nm. The dissociation constants measured from these experiments are summarized in Table 3.2.

**Table 3.2.** Dissociation constants, based on a single site model, for MntR and its mutants with several metal ions as determined by competition titrations against Magfura-2 (MF2) or with ANS dye in protein storage buffer (K\(_d\), with standard deviations).

<table>
<thead>
<tr>
<th></th>
<th>w.t. MntR ANS – K(_d) (μM)</th>
<th>w.t. MntR MF2 – K(_d) (μM)</th>
<th>MntR E99C MF2 – K(_d) (μM)</th>
<th>MntR D8M MF2 – K(_d) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn(^{2+})</td>
<td>92±14</td>
<td>≥50(^a), 160(^b)</td>
<td>≥50(^a)</td>
<td>≥50(^a)</td>
</tr>
<tr>
<td>Co(^{2+})</td>
<td>13±1</td>
<td>4.9±1.3</td>
<td>3.7±0.2</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>Ni(^{2+})</td>
<td>n/d</td>
<td>2.1±0.1</td>
<td>1.3±0.1</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>n/d</td>
<td>0.013±0.003</td>
<td>0.02±0.01</td>
<td>0.03±0.01</td>
</tr>
<tr>
<td>Cd(^{2+})</td>
<td>0.5±0.1</td>
<td>0.1±0.006(^d)</td>
<td>&lt;&lt; 0.016</td>
<td>0.1±0.006</td>
</tr>
</tbody>
</table>

\(^a\) Value provided is a lower limit of the affinity estimated by modeling the data; \(^b\) ~160 μM as measured by EPR spectroscopy T = 25 °C (K\(_{d1}\) = 900 μM and K\(_{d2}\) = 30 μM); \(^c\) 8.87±1.48 as measured by absorption spectroscopy (Figure 3.9); \(^d\) Best fit with a two site model (K\(_{d1}\) = 0.10 μM and K\(_{d2}\) = 3.9 μM).
Figure 3.8. Fluorescence excitation spectra (left) of a competition experiment between MntR (15 μM) and Mag-fura-2 (0.5 μM) titrated with Co$^{2+}$ in protein storage buffer. The initial spectrum is shown as a heavy solid line and the final spectrum is shown as a heavy dashed line (binding of Co$^{2+}$ quenches Mag-fura-2 fluorescence). The spectra represent the emission intensity at 505 nm (ordinate) as a function of excitation wavelength (abscissa). Fit (solid line) of the fluorescence excitation intensity at 380 nm (right) as a function of metal ion concentration. T = 25 °C.

Figure 3.9. UV-Visible titration with MntR and Mag-fura-2 competing for Co$^{2+}$. Electronic absorption spectra (left) of a competition experiment between MntR (50 μM) and Mag-fura-2 (6.5 μM) titrated with Co$^{2+}$ in protein storage buffer. The initial spectrum is shown as a heavy solid line and the final spectrum is shown as a heavy dashed line. Fit of the absorbance at 362 nm (right) as a function of metal ion concentration. T = 25 °C.
Figure 3.10. Fluorescence excitation spectra (left) of a competition experiment between MntR (15 μM) and Mag-fura-2 (0.5 μM) titrated with Cd$^{2+}$ in protein storage buffer. The initial spectrum is shown as a heavy solid line and the final spectrum is shown as a heavy dashed line (binding of Cd$^{2+}$ changes the maxima of Mag-fura-2 fluorescence). The spectra represent the emission intensity at 505 nm (ordinate) as a function of excitation wavelength (abscissa). Fit (two site model, solid line) of the fluorescence excitation intensity at 330 nm (right) as a function of metal ion concentration. T = 25 °C.

Overall, the affinities of MntR appear to loosely follow the Irving-Williams series, with $K_d$ values ranging from 0.01 to $\geq 50$ μM, with Zn$^{2+} <$ Cd$^{2+} <$ Ni$^{2+} \sim$ Co$^{2+} <\ll$ Mn$^{2+}$. The results of the competition titrations between Mag-fura-2 and MntR are in agreement with the findings of the ANS experiments (vide supra). Importantly, the Mn$^{2+}$ affinity could not be measured under the presented conditions; even a >200-fold excess of MntR (110 μM) could not compete with Mag-fura-2 for Mn$^{2+}$ (Figure 3.11). Based on simulations of the data using Dynafit (27, 28), these experiments show that MntR has a surprisingly weak affinity for its cognate metal ion, with a lower limit for the $K_d$ value of Mn$^{2+}$ binding to MntR at $\geq 50$ μM. Indeed, all of the other metal ions tested, including Ni$^{2+}$ and Zn$^{2+}$, which do not effectively activate MntR for DNA binding, have a higher binding affinity for MntR than Mn$^{2+}$. These findings unambiguously
demonstrate that the selective DNA-binding response of MntR is not due to a thermodynamic preference for binding Mn$^{2+}$.

**Figure 3.11.** Mag-fura-2 titration with Mn$^{2+}$ in the presence and absence of MntR. Fluorescence titration of Mn$^{2+}$ into a solution of 0.5 μM Mag-fura-2 (◊) in protein storage buffer. Fluorescence titration of Mn$^{2+}$ into a solution of 0.5 μM Mag-fura-2 and 25 μM MntR (♦) in protein storage buffer. As shown, the titration curves are essentially the same in the absence and presence of protein, indicating MntR does not effectively compete with the dye for Mn$^{2+}$. Normalized fluorescence intensity at $\lambda_{ex}=380$ nm is shown as a function of Mn$^{2+}$ concentration. $T = 25$ °C.

Surprisingly, similar to wild type MntR, both the MntR E99C and the MntR D8M mutants still exhibit metal-binding affinities in the micromolar range (Table 3.2). When compared to wild type MntR, metal affinities of mutant proteins still follow the Irving-Williams series and display the weakest affinity for Mn$^{2+}$. Furthermore, mutant MntR affinities for Ni$^{2+}$, Co$^{2+}$ and Zn$^{2+}$ appear almost identical to those of wild type protein. One significant difference is the noticeable increase in Cd$^{2+}$ affinity of the MntR E99C mutant. Under conditions employed in our experiments, Mag-fura-2 proved
unable to compete with MntR E99C (no changes in Mag-fura-2 fluorescence were observed until MntR E99C was loaded with Cd\(^{2+}\), data not shown). Because the dye was unable to compete with the protein for Cd\(^{2+}\), we estimate the upper limit of MntR E99C affinity for Cd\(^{2+}\) at 0.016 μM, based on Mag-fura-2 affinity of this metal ion.

**EPR Spectroscopy**

Samples of 0.25 mM apoMntR were titrated with MnCl\(_2\) in successive additions up to approximately 6 equivalents. The room temperature EPR spectra of the titration showed a 6-line hyperfine pattern typical of \(^{55}\text{Mn}^{2+}\) (inset of Figure 3.12). This spectrum is typical of Mn(H\(_2\)O\(_6\))\(^{2+}\) species. All titration points showed the same spectra, but with varying intensity of the signal. The concentration of Mn(H\(_2\)O\(_6\))\(^{2+}\) in the titration series was determined by normalizing the intensity to a sample of a known amount of MnCl\(_2\) added to the same buffer and recorded under the same conditions. The amount of Mn\(^{2+}\) detected in these spectra was a fraction of the total amount added to the protein sample, indicating binding of Mn\(^{2+}\) to the protein is an equilibrium process. Mn\(^{2+}\) complexed to protein reduces the coordination of weak-field water, increasing spin-orbit interactions and zero-field energies relative to that of Mn(H\(_2\)O\(_6\))\(^{2+}\). Consequently, the signals from the protein bound Mn\(^{2+}\) species are too broad to be detectable at room temperature (29). A plot of the Mn(H\(_2\)O\(_6\))\(^{2+}\) equivalents observed versus the total equivalents of Mn\(^{2+}\) added to the samples is shown in Figure 3.12. The results were repeated for two independent titration experiments.
Figure 3.12. Equivalents (open circles) of the Mn(H$_2$O)$_6^{2+}$ species versus equivalents of added Mn$^{2+}$, as measured from the intensity of the room temperature EPR spectra. The mathematical curves show the equivalents of the Mn(H$_2$O)$_6^{2+}$ species (solid line) and the protein bound binuclear Mn$^{2+}$ species (dashed line) generated from the sequential binding model of Eq. 3.4 with $K_{d1} = 900$ μM, $K_{d2} = 30$ μM. Inset: room temperature EPR spectrum of 0.19 mM MntR with 1.15 mM MnCl$_2$ added. Spectral conditions: microwaves, 9.78 GHz, 20 mW; modulation, 0.1 mTpp.

The equivalence of Mn(H$_2$O)$_6^{2+}$ data can be fit to the sequential metal binding model of equation 3.4. The theoretical curve for equivalents of Mn(H$_2$O)$_6^{2+}$ (Figure 3.12) is shown for $K_1K_2 = 4(1)x10^7$ M$^{-2}$. The calculated difference (total Mn$^{2+}$ minus Mn(H$_2$O)$_6^{2+}$) is also shown in Figure 3.12 and displayed in terms of equivalents of binuclear Mn$^{2+}$ sites. Significantly, the difference asymptotically approaches 2 equivalents, indicating a stoichiometry of four Mn$^{2+}$ ions bound to MntR. The specific amounts of protein bound metal species are not directly measurable; consequently, the theoretical curve is most sensitive to the product $K_1K_2$ of the equilibrium constants and to a lesser extent the ratio $K_2/K_1$. The data can be equally well fit with $K_1K_2$ within the uncertainty of the value given. These data also require $K_2/K_1 > 5$, and the low
temperature data to follow will require $K_2/K_1 > 30$. For $K_2/K_1 = 30$, the corresponding dissociation constants are $K_{d1} = 900 \mu M$ and $K_{d2} = 30 \mu M$.

For low temperature experiments, samples of 0.25 mM MntR were titrated with MnCl$_2$ in successive additions up to approximately 6 equivalents. Figure 3.13 shows EPR spectra of this titration at temperatures of 2 and 14 K. All spectra are plotted as signal×temperature (the intensity scale of the spectrum is multiplied by the temperature). For such plots, signals that display Curie law dependence (intensity ~ 1/T) will show the same intensity. The sharp six-line pattern observed at $g = 2.0$ is typical of Mn(H$_2$O)$_6^{2+}$, and as expected, the temperature dependence of this signal is strictly proportional to 1/T. In contrast, the broader wings of the spectra near $g = 2.5$ and 1.7 grow in with increasing temperature. At higher temperatures (data not shown), the spectra are nearly the same as that of the 14 K data when plotted as signal×temperature. EPR signal intensities of Mn$^{2+}$ species that are not proportional to 1/T are indicative of the presence of a spin interaction between the Mn$^{2+}$ ions.
Figure 3.13. EPR spectra of MntR titrated with MnCl₂, recorded at 14 K (solid lines) and 2.3 K (dashed lines). The intensity of the spectra are plotted as signal × temperature after normalization for instrumental parameters. Additional scaling of spectra at both temperatures are as shown on the figure. The nominal concentrations of MntR was 0.22 mM. The total MnCl₂ added, in equivalents, is as noted for each spectrum. Spectral conditions; microwaves, 9.65 GHz, 0.2 mW (14 K) and 0.02 mW (2.3 K); modulation amplitude 1.0 mT.pp.

An isolated Mn²⁺ center with an axial zero-field splitting parameter D ≤ 0.1 cm⁻¹ will have energies of all spin levels < 0.6 cm⁻¹. Our calculations indicate that the populations of all spin levels will be roughly equal for temperatures down to 2 K; thus the intensity of the spectra will be proportional to 1/T for temperatures >2 K (Curie law). For D > 0.1 cm⁻¹, the EPR spectra would have discernable features well outside the g = 2 region (30), which is not true for MntR. The temperature variation for MntR is subtle overall, but clearly different from control samples. EPR spectra of samples of Mn²⁺ in water, various buffers, and when bound to other proteins, all show spectra with exact 1/T dependence of signal intensities from temperatures of 2 K and higher, without
deviations from 1/T in the wings of the spectra as is observed here for MntR. Thus, the signals at g = 2.5 and 1.7 originate from a binuclear Mn$^{2+}$ site. The weak temperature dependence and lack of significant features is not common in previously characterized Mn$^{2+}$ complexes. Binuclear Mn$^{2+}$ complexes usually show signals that change over a much wider temperature range and are often identified by an 11-line hyperfine pattern. The reason for the lack of temperature dependence and discernable signal features is due to a weaker exchange interaction than is typical of most Mn$^{2+}$ complexes or proteins (vide infra), resulting in a smearing of the hyperfine patterns. As the Mn$^{2+}$ concentration is increased, the signal from the Mn(H$_2$O)$_6^{2+}$ species dominates the spectra, and the temperature dependence is then proportional to 1/T. The signals and temperature dependencies were not affected by a variety of different buffer conditions: pH 8.5, 30% glycerol, or 600 mM NaCl. Rapid freezing of the samples in cold isopentane (-140 °C) also had no effect on the spectra. The addition of excess orthovanadate, which is known to bind to some binuclear Mn$^{2+}$ proteins (31), also did not effect the spectra.

The parallel mode EPR of MntR show signals from two different species (Figure 3.14). At low equivalents, a broad featureless signal appears at g = 6.7 which increases in intensity proportionately and displays the same non Curie law behavior as the binuclear Mn$^{2+}$ signal in perpendicular mode. At higher equivalents, a new signal grows in the same region with a 6-line hyperfine pattern. The intensity of this signal is proportional to the Mn(H$_2$O)$_6^{2+}$ signal in perpendicular mode. At 14 K the spectrum at larger equivalents is dominated by free Mn$^{2+}$ and is unresolvable from the binuclear
Mn$^{2+}$ signal. Due to the overlap of these two signals and low signal-to-noise, the parallel mode signals will not be discussed further.

**Figure 3.14** Parallel mode EPR spectra of MntR at 2.3 K. [MntR] = 0.22 mM. The total MnCl$_2$ added, in equivalents, are as noted. Spectra are scaled for equal concentrations of Mn$_2$-MntR in sample; 0.13 mM (A) and 0.42 mM (B). Spectral conditions; microwaves 9.34 GHz, 0.2 mW; modulation amplitude 1.0 mT$_{pp}$.

The low temperature EPR spectra at the lowest and highest equivalents of total Mn$^{2+}$ show differing, but always nonzero, contributions of Mn(H$_2$O)$_6^{2+}$ and the binuclear Mn$^{2+}$ protein species. Thus, direct observation of signals from the corresponding pure species is not possible. Consequently, we employ the method of Singular Value Decomposition (SVD) to the EPR titration spectra to determine the product binding constant, $K_1K_2$, in accordance with equation 3.4. The SVD method allows
decomposition of a series of spectra into the corresponding spectra of the pure species, usually referred to as base spectra. Important tests for the validity of the SVD method on the particular application are: (1) eigenvalues for the base spectra which are significantly greater than the eigenvalues of the spectra representing noise, and (2) base EPR spectra which are representative of physically correct EPR spectra. The SVD method was applied to the 14 K data shown in Figure 3.15. The resulting base spectra are shown in Figure 3.16A and 3.16B.

**Figure 3.15.** EPR spectra of MntR titrated with MnCl₂ recorded at 14 K (solid lines) and SVD reconstructed spectra (dashed lines) for $K_1K_2 = 5 \times 10^8$ M⁻². Additional scaling of spectra are as shown on the figure. The nominal concentrations of MntR was 0.22 mM. The total MnCl₂ added, in equivalents, is as noted for each spectrum. Spectral conditions; microwaves, 9.65 GHz, 0.2 mW; modulation amplitude 1.0 mTpp.
Figure 3.16. EPR basis spectra derived from the SVD decomposition of the spectra of the titration of MntR with MnCl₂: (A) Mn(H₂O)₆²⁺, (B) Mn₂-MntR. The basis spectra are derived using the consecutive binding model (Eq. 3.4) for the 14 K spectra, and \( K_1K_2 = 5 \times 10^8 \text{ M}^{-2} \). A 1:1 addition of the (A) and (B) gives the 1.2 eq EPR spectra at 14 K of Figure 3.13. (C) Simulations of the Mn₂-MntR species at temperatures of 14 K (solid line) and 2.3 K (dashed line). The intensity scale of the 14 K simulation is calculated for 0.13 mM Mn₂-MntR (fully load protein). Spectrum B is plotted for this same scale. The intensity of 2.3 K simulation is scaled for \( 1/T \) relative to the 14 K simulation. Simulation parameters are for two identical Mn²⁺ ions: \( J = -0.2 \text{ cm}^{-1} \) (-2JS₁•S₂), S=5/2, I=5/2, A=250 MHz, D=0.04 cm⁻¹, E/D=0.21, \( r_{\text{MnMn}} = 4.4 \text{ Å} \), \( \theta = 45° \).

The eigenvalues for these base spectra were more than an order of magnitude greater than the eigenvalues for spectra at the noise level, indicating the existence of only two significant spectral species. In addition, the base spectra have features that are attributable to (A) binuclear (see below) and (B) Mn(H₂O)₆²⁺ species. The intensity of the base spectra is displayed for the decomposition of the 5.4 equivalents sample at 14 K. The sum of the two basis spectra for the range of the titration data, with relative amounts determined by the model of equation 3.4, is shown in Figure 3.15. The SVD
method gave a product binding constant of $K_1K_2 = 5(5) \times 10^8 \text{ M}^{-2}$. Values of $K_1K_2$ outside of the uncertainty gave meaningless base spectra and significantly poorer fits to the titration spectra. Two independent titrations gave similar results. For these data, we have the additional benefit over the room temperature data of direct detection of a protein bound binuclear Mn$^{2+}$ species. This allows a better determination of the ratio $K_2/K_1 > 30$. For ratios less than this, the amount of monomeric Mn signal would be not be compatible with the data. For $K_2/K_1 = 30$, the corresponding dissociation constants are $K_{d1} = 250 \mu\text{M}$ and $K_{d2} = 8 \mu\text{M}$. The values determined from the low temperature titration are slightly smaller than those at room temperature, indicating tighter binding of Mn$^{2+}$ to the protein upon freezing of the samples. At low equivalents of Mn$^{2+}$, the SVD approximated spectra show a poorer fit to the data than at higher equivalents. This discrepancy is possibly due to a protein bound mononuclear Mn species at low equivalents. SVD was applied to the data using the 3 species model, where the third species is a bound mononuclear Mn$^{2+}$ species; however, no convergence to the experimental spectral set could be found. We suspect that the SVD extraction of a mononuclear Mn$^{2+}$ spectral species, apart from that of free Mn$^{2+}$, will require many more titration points in the low equivalent regime.

The type of binuclear Mn$^{2+}$ spectrum observed from MntR (Figure 3.13), to our knowledge, has no precedent in the literature. In previous work, we have shown new methods that now allow quantitative simulation of complicated spectra from Mn$^{2+}$ dimers (32) as a function of temperature, microwave frequency, and microwave orientation. The complexes of this previous study have sufficiently large spin exchange ($J \gg D$, $g\beta B$), to give well isolated excited spin manifolds and corresponding subspectra.
from each manifold. For MntR, the spin exchange energy is small, thus the splitting between the spin manifolds is comparable to the zero-field and Zeeman energies. Consequently, there are no isolated spin manifolds or subspectra, the spin levels all mix, and we expect that the many overlapping transitions will produce a broad featureless spectrum. The spectrum should have greatest intensity near $g = 2$, since the transitions with the highest probability occur here. In addition, at temperatures as low as 14 K, each spin level has nearly equal population, and we expect the spectra should show Curie law behavior.

Figure 3.16C shows simulations of the MntR binuclear Mn$^{2+}$ site at temperatures of 2 and 14 K. The simulated spectra were calculated for equivalent Mn$^{2+}$ sites (parameters given in Figure 3.16). As we have demonstrated in our previous work, the software allows quantitative comparisons of experimental spectra with simulations. From the SVD results, the concentration of the binuclear species in Figure 3.16B is $[\text{Mn}_2\text{-MntR}] = 0.13$ mM. The simulations of Figure 3.16C are calculated for the same concentration of the binuclear Mn$^{2+}$ species. While the simulation does not match all of the features of the spectrum, the intensity is in approximate agreement with that of the experimental spectrum. The experimental spectra do not display sufficient resolution to allow determination of any of these parameters with certainty, except for the exchange interaction. The assignment to a binuclear species is unambiguous. For an antiferromagnetic exchange coupling of $J = -0.20(5)$ cm$^{-1}$ ($H = -2JS_1\bullet S_2$) for the binuclear Mn$^{2+}$ site, the temperature dependence of the simulations matches that of the experimental data shown in Figure 3.13. The temperature dependence of the data
cannot be reproduced with only a magnetic dipolar interaction between the Mn\(^{2+}\) ions, since this represents an energy contribution to the system of less than 0.05 cm\(^{-1}\).

**Fluorescence Anisotropy Experiments**

To get a better idea of how the metal binding of MntR affects DNA binding, we revisited our prior fluorescence anisotropy experiments, which were previously performed in the presence of 1.0 mM metal ion (11, 17), which was in excess of >100-fold over the concentration of MntR. To probe the effect of metal ion affinity, the binding of MntR to the consensus sequence oligonucleotide dsmntH26 was measured under lowered metal ion concentrations. The binding of MntR to dsmntH26 was unchanged when the concentration of Mn\(^{2+}\), Co\(^{2+}\), or Cd\(^{2+}\) was dropped from 1.0 mM to 100 \(\mu\)M. In contrast, at 10 \(\mu\)M metal ion (Figure 3.17), the binding of MntR to dsmntH26 was preserved with Cd\(^{2+}\), but was virtually abolished with Mn\(^{2+}\). In the presence 10 \(\mu\)M Co\(^{2+}\), MntR binding to dsmntH26 is diminished, but not totally eradicated. These observations are wholly consistent with the measured metal binding affinities; at 10 \(\mu\)M M\(^{2+}\), the protein can still bind Cd\(^{2+}\) and to a lesser degree Co\(^{2+}\) because the amount of available metal exceeds the measured \(K_d\) values (Table 3.2). In contrast, 10 \(\mu\)M Mn\(^{2+}\) is below the \(K_d\) value for this metal ion leading to loss of DNA binding ability. The observed effects of lowered metal ion concentrations on the DNA binding ability of MntR are in good qualitative agreement with the measured metal binding affinities of MntR.
Figure 3.17. DNA binding assays with 10 nM dsmntH26 titrated with MntR in presence of 1000 μM (O), 100 μM (■), or 10 μM (×) of metal ion. Results are shown for Cd²⁺ (top), Co²⁺ (middle), and Mn²⁺ (bottom). Buffer = 20 mM HEPES pH 7.2 @4 °C, 500 mM NaCl, 5% (v/v) glycerol. T = 25 °C.
Our prior work with MntR has focused on elucidation of the metal-mediated DNA binding properties of this protein (11, 17) and studies on how metal binding affects protein structure (17). Two new reports describe some metal binding affinities of MntR and a homolog AntR (14, 18); our results are compared with these recent findings below. Measuring the metal binding affinities is an essential component for understanding how MntR selectively responds to its cognate metal ion and elucidating its mechanism of action.

In preliminary experiments to gauge the metal binding affinities of MntR, the hydrophobic dye ANS was utilized as an exogenous fluorescent reporter. Changes in ANS fluorescence were used as an indicator of metal induced structural organization; $K_d$ values could be estimated based on changes in ANS fluorescence intensity as the dye was excluded from hydrophobic surfaces on MntR. The $K_d$ values determined by this method were 92 $\mu$M for Mn$^{2+}$, 13 $\mu$M for Co$^{2+}$, and 0.5 $\mu$M for Cd$^{2+}$ (Table 3.2). These results were the first to indicate that MntR does not show a tight binding for its cognate metal ion Mn$^{2+}$. ANS is an environmental probe and hence its fluorescence depends on the interaction of the dye with hydrophobic portions of MntR. As the location of binding, binding affinity, and number of binding sites for ANS on MntR are not known, the values determined by this approach are subject to several sources of error/bias (e.g. ANS might compete for the metal binding sites), and the $K_d$ values obtained are best interpreted as relative affinities. With these limitations in mind, an alternative method to corroborate the values obtained from the ANS experiments was sought.
Competition titrations between the protein and a spectroscopically active competitor ligand were selected as a means to quantitatively measure the metal binding affinities of MntR. Fura-2 and Mag-fura-2 are fluorescent dyes that have been previously employed for studying other metalloregulatory proteins (6, 26) and thus were selected as a starting point for our investigation. Both dyes are frequently employed as ratiometric Ca\(^{2+}/Mg\(^{2+}\) chelators, but can also bind transition metal ions with related spectral changes. The affinities of Fura-2 for several transition metal ions has been determined (19-22), while for Mag-fura-2 only the binding constant with Zn\(^{2+}\) has been reported (24).

Fura-2 was selected for initial experiments because its affinities for Mn\(^{2+}\), Fe\(^{2+}\), Co\(^{2+}\), and Cd\(^{2+}\) ions are known and are in a range (pM to nM) observed for many metalloregulatory proteins (33). Surprisingly, in our competition experiments between Fura-2 and MntR, where MntR was present in a 10-fold excess over Fura-2, MntR proved unable to compete for the metal ions Mn\(^{2+}\), Co\(^{2+}\), and Cd\(^{2+}\). Based on the inability of MntR to compete with Fura-2 for binding these metal ions, an approximate lower limit on the $K_d$ values for MntR could be inferred: Mn\(^{2+}\) > 30 nM, Co\(^{2+}\) > 90 nM, and Cd\(^{2+}\) > 0.01 nM. Compared to Fura-2, Mag-fura-2 lacks several ligating atoms (Figure 3.4) and has Ca\(^{2+}/Mg\(^{2+}\) affinities two orders of magnitude weaker than that of Fura-2 (20, 23). Because the binding affinities of Fura-2 for transition metal ions are several orders of magnitude stronger than they are for alkali earth metals, it was anticipated that Mag-fura-2 would show a similar binding constant trend (e.g. $K_d$ values for transition metal ions in the nM to \(\mu\)M range). Previously, Zn\(^{2+}\) was the only transition metal ion for which the binding constant with Mag-fura-2 had been reported.
Therefore, the stoichiometry and metal binding affinity of Mag-fura-2 for Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$ (as a control), and Cd$^{2+}$ were measured (at $I = 0.11$ M and 25 °C) using the approach originally employed for determining Fura-2 affinities for Mn$^{2+}$ and Fe$^{2+}$ (20). Specifically, Fura-2 fluorescence quenches upon binding to these paramagnetic ions, and the binding affinities can be determined by monitoring this quenching as the transition metal ions compete with Ca$^{2+}$ for the dye. Mag-fura-2 shows similar fluorescence behavior, with Mn$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ quenching the fluorescence of the dye and Zn$^{2+}$ and Cd$^{2+}$ exhibiting fluorescence spectra similar to that generated upon binding Ca$^{2+}$. Based on this observation, the affinities of Mag-fura-2 for paramagnetic ions were measured by competition against Ca$^{2+}$, while affinities for Zn$^{2+}$ and Cd$^{2+}$ were measured by competition against the paramagnetic ions (after the $K_d$ values were determined). As anticipated, the measured $K_d$ values (Table 3.1) fall in the low μM to nM range, which should prove useful in studies involving relatively weak metal-ligand or metal-biomolecule interactions.

### Metal Binding Affinities of MntR

After determining Mag-fura-2 affinities for several transition metal ions, dye-protein competition titrations were performed to determine metal binding affinities of MntR. Overall, the binding behavior loosely follows the Irving-Williams series (34), with $K_d$ values (Table 3.2) following the order Zn$^{2+} <$ Cd$^{2+} <$Ni$^{2+} \sim$ Co$^{2+} \ll$Mn$^{2+}$. The model used to evaluate the metal binding of MntR utilizes only a single binding constant; however, crystallographic analysis of MntR loaded with Mn$^{2+}$, Co$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$ show that only Mn$^{2+}$ and Cd$^{2+}$ form binuclear sites (12, 14), while MntR binds
only one Co\(^{2+}\) or Zn\(^{2+}\) ion per monomer (14) (A. Glasfeld personal communication). Attempts to analyze Cd\(^{2+}\) binding to MntR with a two site model was found to improve the fit to the data (\(K_{d1} = 0.10 \mu\text{M}\) and \(K_{d2} = 3.9 \mu\text{M}\)), and the \(K_d\) values are provided as a footnote in Table 3.2. Under our present experimental conditions, this two binding site analysis for Cd\(^{2+}\) is the most robust model of the data we can provide. The weak binding affinity of MntR for Mn\(^{2+}\) determined by competition with Mag-fura-2 qualitatively agrees with the measured dissociation constants determined by ANS dye experiments and EPR data presented here (vide infra), as well as those determined by EPR for the homolog AntR (18). In contrast, the dissociation constant determined in a recent report by calorimetry (~3 \(\mu\text{M}\) for two independent sites, vide infra) (14) is significantly stronger and does not agree with either of the aforementioned studies. Furthermore, the dissociation constants measured by ITC between MntR and Zn\(^{2+}\) (2 – 6 \(\mu\text{M}\)) and Cd\(^{2+}\) (7 – 17 \(\mu\text{M}\)) are ~300- and 180-fold larger than those determined here by competition with Mag-fura-2 (14). The slightly higher affinity of MntR for Mn\(^{2+}\) versus Cd\(^{2+}\) reported by ITC is inconsistent with the DNA-binding data reported here, where in the presence of low concentrations of Cd\(^{2+}\) MntR is competent to bind DNA, while at the same concentration of Mn\(^{2+}\) DNA binding is completely abolished (Figure 3.17). Differences in the buffer conditions for the ITC versus the experiments reported here (500 mM NaCl, pH 8, 10% for ITC vs 200 or 300 mM NaCl, pH 7.2, 5% glycerol) seem inadequate to explain the disparate findings from these two studies. The ITC experiments were reported in large part to confirm the metal:protein monomer binding stoichiometry (2:1 for Mn\(^{2+}\) and Cd\(^{2+}\), 1:1 for Zn\(^{2+}\)) observed crystallographically (14). Based on the aforementioned discrepancies, we propose that the ITC values are a useful
gauge of binding stoichiometry, but that the Mag-fura-2 and EPR studies presented here are a more reliable determination of the binding constants.

The EPR study of AntR reported a binding affinity of this protein for Zn$^{2+}$ of 152±47 μM based on a 2:1 binding stoichiometry per AntR monomer with infinite cooperativity between the binding sites (18). This value is substantially different than the $K_d$ value of 13±3 nM determined here for MntR and Zn$^{2+}$ based on a single binding site. The disparity between these findings is surprising, considering the good agreement for the Mn$^{2+}$ $K_d$ values and the extremely high homology between these two proteins. Both AntR and MntR are 142 aa in length and an alignment analysis (ClustalW v1.83, data not shown) shows that the proteins share 82% sequence homology and all of the metal binding residues in MntR are conserved in AntR. Again, the solution conditions of these experiments are insufficient to explain the large difference in the Zn$^{2+}$ $K_d$ values. At the moment, the difference in the studies is puzzling; however, the activation profile for AntR may provide an explanation. For AntR, it is reported that the Zn$^{2+}$ form is a good analog of the Mn$^{2+}$ bound form, with the former being activated for binding the cognate DNA sequence (18). This finding is in sharp contrast to MntR, for which Zn$^{2+}$ is an extremely poor activator of DNA binding (11). This comparative evidence suggests that despite the very high homology of these two proteins, they may well have different activation profiles and hence different metal binding sites. This explanation remains highly speculative and further biophysical and structural characterization of AntR is required to resolve this apparent inconsistency in the AntR/MntR findings.
Metal binding affinities of MntR mutants

In vivo MntR mutant studies had shown that introduction of thiol residues into the MntR metal site can result in loosened metal specificity (35). In particular, if the D8M mutation was present, MntR could respond to both Fe\textsuperscript{2+} and Mn\textsuperscript{2+}, although Mn\textsuperscript{2+} was still the preferred activator (35). This trend was further reflected in our experiments with both mntH and mntA recognition sequences in vitro (17, chapter 2 of this thesis). In continuation of our prior work with MntR D8M and MntR E99C mutants, we measured the metal binding affinities of these proteins to investigate whether these mutations alter metal selectivity of MntR. In comparison to wild type MntR, MntR D8M and MntR E99C metal affinities still follow the Irving-Williams series and fall in low to sub-micromolar K\textsubscript{d} range. Affinities for Ni\textsuperscript{2+}, Co\textsuperscript{2+} and Zn\textsuperscript{2+} remain largely unaltered for both mutants, while Mn\textsuperscript{2+} affinities were too weak to be determined by competition with Mag-fura-2. In case of Cd\textsuperscript{2+}, affinity of MntR D8M mutant appears unperturbed, while significant increase in MntR E99C affinity is observed. The observed metal affinities are particularly startling in light of the DNA-binding affinities observed with these mutants (17, chapter 2 of this thesis). In particular, while the mutant metal affinities appear largely unperturbed, the DNA affinities showed significant difference depending on the nature of mutant (17, chapter 2 of this thesis).

A possible explanation for the lack of significant differences in the observed metal binding affinities for Ni\textsuperscript{2+}, Co\textsuperscript{2+} and Zn\textsuperscript{2+} ions between wild type and mutant MntR proteins may be the change in metal ion coordination. While no crystallographic data is available on Ni\textsuperscript{2+}-MntR, crystallographic evidence has shown that akin to Mn\textsuperscript{2+}-MntR D8M, wild type MntR forms mononuclear site A with both Zn\textsuperscript{2+} and Co\textsuperscript{2+} (Figure
3.2) (A. Glasfeld personal communication, (14)). It is plausible that the mutant proteins still form a mononuclear site, but employ a slightly different coordination geometry. While new coordination geometry may not significantly alter the metal-binding affinities, the impact on the MntR structure could result in different DNA-binding affinities. This proposed change in coordination geometry could either result from the metal ion binding to site C or from formation of a new site that employed ligands from both sites A and C (Figure 3.2). Regrettably, at the moment crystallographic data is not available to support this assumption and further structural work is necessary to fully explain the nature of the discrepancy between the MntR mutants metal-binding and DNA-binding affinities.

Interestingly, Cd$^{2+}$ affinity of MntR E99C mutant appears to correlate well with the thermal melting experiments that gauged how much a metal ion stabilized the structure of this protein ((17), chapter 2 of this thesis). In particular, in case of the thermal melting experiments, MntR E99C exhibited higher melting temperatures than wild type MntR in presence of Cd$^{2+}$, suggesting that this ion is bound tighter by the mutant protein. As expected, this mutation shows that introduction of the soft, thiol-based ligand into the protein results in stronger metal binding to a soft metal ion.

**EPR Spectroscopy**

The crystal structure of MntR identifies four binding sites for Mn$^{2+}$ per protein dimer, which forms two binuclear Mn$^{2+}$ centers. To date, no other direct spectroscopic probe of the electronic environment of the metal has been available. Here we report the first EPR spectra to show direct evidence for the quantitative formation of a binuclear
Mn$^{2+}$ center from solution samples. The temperature dependent data and simulations indicate a weak antiferromagnetic exchange coupling of $J = -0.2 \text{ cm}^{-1}$. The exchange interaction for binuclear Mn$^{2+}$ model complexes with H$_2$O/dicarboxylato bridges are in the range -1 to -3 cm$^{-1}$ (36, 37), and hydroxo-/dicarboxylato bridges are -9 cm$^{-1}$ (38). Binuclear Mn$^{2+}$ model complexes with only dicarboxylate bridges have exchange couplings near -1 cm$^{-1}$ (39-41). The exchange coupling for MntR is significantly smaller than that of binuclear Mn$^{2+}$ centers in these model complexes. A binuclear Mn$^{2+}$ model complex with a single carboxylato bridge has been characterized with $J = -0.19 \text{ cm}^{-1}$ (42); a value close to that for MntR.

The crystal structures of MntR identify two protein conformations that bind Mn$^{2+}$ with differing protein and solvent coordination. The structure with a Mn···Mn distance of 3.3 Å with a bridging hydroxyl species would give characteristically different EPR spectra and a larger exchange value than observed for MntR in these studies. The other structure has a significantly longer Mn-Mn distance of 4.4 Å. For this structure, the manganese ions are bridged by $\mu_{1,3}$-carboxylato-(Glu99) and $\mu$(O)-carboxylato-(Glu102) in two different configurations. In both configurations, both carboxylato groups have a long Mn-O bond (~2.5 Å). These long bonds will weaken the exchange pathway, which is consistent with the lower exchange value observed here for MntR. We have made many attempts to facilitate the formation of the structure with the shorter Mn···Mn distance. We found no change in the binuclear Mn$^{2+}$ complex at pH 7.2 and 8.5. In addition, higher salt levels, a cryo-protectant (glycerol), and flash-freezing of protein all had no effect on the signals. These results suggest a role for
crystal packing forces in the structure with the shorter Mn\textperiodcentered Mn distance, and that the structure with the longer Mn\textperiodcentered Mn distance is relevant in solution studies.

The ratio of the binding constants indicates that the binding of the first Mn\textsuperscript{2+} to MntR is significantly weaker than that of the second Mn\textsuperscript{2+}. This ratio indicates a cooperative binding process which is perhaps better described by the overall equilibrium for the binding of two Mn\textsuperscript{3+} ions (2Mn\textsuperscript{2+} + apoMntR \leftrightarrow Mn\textsubscript{2+}-MntR) with an equilibrium constant $K = K_1K_2$. Our dissociation constants ($K_{d1} = 900 \ \mu M$, $K_{d2} = 30 \ \mu M$, $\sqrt{K_d} = 160 \ \mu M$) are comparable to those determined previously from a AntR ($K_{d1} = 210 \ \mu M$ and $K_{d2} = 17 \ \mu M$, $\sqrt{K_d} = 60 \ \mu M$) (18), but significantly greater than those obtained from ITC measurements ($K_{d1} = 10 \ \mu M$ and $K_{d2} = 1 \ \mu M$, $\sqrt{K_d} = 3 \ \mu M$) (14). The value of $\sqrt{K_{d1}K_{d2}}$ is more certain than the individual values, and are also consistent with the results of the ANS and Mag-fura-2 experiments reported here.

The EPR measurements detect a small change (factor of 4) towards tighter binding upon freezing MntR. The crystallographic data finds two different conformations of the Mn\textsubscript{2} site, that depend on crystallization conditions, having a short (3.3 Å) and long (4.4 Å) Mn\textperiodcentered Mn distance (14). We do not attribute the change in the binding constant to a switch between these conformations, for the following two reasons. First, the increase in the stability of the complex upon freezing is in the range expected for the enthalpy change due to metal-ligand binding. For example, the enthalpy change for binding of Mn\textsuperscript{2+} to two histidines is -5.2 kcal/mole (43). A change in temperature from 25 °C to 0 °C will increase the stability of the complex by a factor
of 2. Second, the EPR spectra of samples that were frozen over 1 minute were identical to spectra of flash frozen samples. If the interconversion between the two conformations (e.g. short Mn⋯Mn at room temperature, long Mn⋯Mn when frozen) is due to a relatively slow protein dependent event, then the flash frozen samples should have shown a significant change in the EPR spectrum. Thus, we conclude that the change in the binding constant is an enthalpic effect rather than a change in Mn⋯Mn distance.

**Metal Affinities of MntR versus Other Metalloregulatory Proteins**

Recent calorimetry studies on DtxR were able to dissect a high \((2 \times 10^{-7} \text{ M})\) and low \((6.3 \times 10^{-4} \text{ M})\) affinity site for the binding of two \(\text{Ni}^{2+}\) ions per protein monomer \((10)\); however, in DtxR the metal binding sites are well separated and possess substantially different ligating residues, a factor likely contributing to the measurable difference in affinities. In contrast, \(\text{Mn}^{2+}\) binding to MntR is best fit with a cooperative binding model. The coordination environments of the two metal ions in the binuclear site of MntR are very similar: both are essentially hexacoordinate with one nitrogen and five oxygen based ligands \((12, 14)\). The proximity of the two sites also supports the observation that the binding of \(\text{Mn}^{2+}\) is cooperative. Similarly, metal binding was also found to be highly cooperative in AntR \((18)\), further supporting the cooperative binding model found for \(\text{Mn}^{2+}\) binding to MntR.

The observation that metal binding in MntR generally follows the Irving-Williams series \((34)\) is not unique to this metalloregulatory protein. Similar findings have been obtained with Fur, NikR, and NmtR, which have completely different metal
response profiles from MntR \((6, 7, 44)\). The observation that several classes of metalloregulatory proteins show similar metal binding trends clearly indicates that selective allosteric regulation is not merely a reflection of metal binding affinity. This is particularly noteworthy for MntR as the Irving-Williams series dictates (and our data support) that the cognate metal ion \(\text{Mn}^{2+}\) will be bound with lower affinity than most other biologically relevant, divalent transition metal ions. This finding shows that the selectivity of MntR must be of a different origin, which likely involves a critical active site geometry imposed by \(\text{Mn}^{2+}\) \((14)\) or perhaps specific \(\text{Mn}^{2+}\) delivery via a metallochaperone \((45)\), consistent with the arguments of Robinson and Giedroc \((44-46)\).

With the metal-binding affinities of MntR determined, we can now re-evaluate the selective activation of MntR for DNA binding. As already detailed, the cognate metal ions, \(\text{Mn}^{2+}\) and \(\text{Cd}^{2+}\), are bound quite weakly and tightly, respectively. \(\text{Co}^{2+}\), an ion that leads to modest DNA binding at saturating levels, as well as \(\text{Ni}^{2+}\) and \(\text{Zn}^{2+}\), which do not effectively activate DNA binding even at saturation, are all bound by MntR more tightly than \(\text{Mn}^{2+}\). This indicates that although MntR is competent for binding \(\text{Co}^{2+}\), \(\text{Ni}^{2+}\), and \(\text{Zn}^{2+}\), the metals do not induce the appropriate allosteric change to induce tight DNA binding. In the case of \(\text{Co}^{2+}\) and \(\text{Zn}^{2+}\), incomplete activation is in part due to failure to form a binuclear site \((14)\) (A. Glasfeld, personal communication). \(\text{Ni}^{2+}\) may also fail to form a binuclear site, or the geometry of such a site with this metal ion may be significantly different, which again fails to produce the requisite allosteric change in MntR. Overall, we would concur with the hypothesis of Glasfeld et al. \((14)\) that the geometry and stoichiometry (binuclear) of metal binding to MntR is more significant to the mechanism of activation than metal ion affinity.
It is interesting that Fur and DtxR family members exhibit metal affinities generally much weaker than members of MerR and ArsR/SmtB families, the latter of which generally bind cognate ions in the $10^{-9}$ to $10^{-21}$ M range (8, 26, 33, 47-52). One explanation for this apparent trend may come from considering the function of these metalloregulatory protein families. MerR and ArsR/SmtB metalloregulators generally regulate genes (as repressors in their apo form) required to export toxic metal ions or an overabundance of an otherwise essential metal ion (33, 47). The removal of such harmful metal ions may require a very sensitive response to manage the threat before damage is incurred on the cell. In contrast, Fur- and DtxR-family proteins are generally responsible for controlling genes involved in essential metal ion import (acting as repressors in their holo forms) (53). Because cellular acquisition of an essential metal ion may be more tolerant to a slight excess than a deficiency of the ion, the proteins that regulate metal ion influx may be responsive at much higher metal concentrations in order to ensure that adequate metal stores are obtained before suppressing import genes. In this context, one can rationalize why members of Fur/DtxR families have weaker cognate metal binding affinities when compared to MerR/ArsR families.

The aforementioned hypothesis may also be an argument for contextualizing the difference in binding affinities of MntR for Mn$^{2+}$ and Cd$^{2+}$. It is known that several Mn$^{2+}$ transporting proteins are capable of also transporting Cd$^{2+}$ (54). At saturation concentrations, both Mn$^{2+}$ and Cd$^{2+}$ were found to be equally strong activators of MntR for DNA binding (11); however, the studies here show that MntR binds Mn$^{2+}$ at least 1000-fold less tightly than Cd$^{2+}$. Therefore, in presence of Mn$^{2+}$, MntR functions, as expected, like a member of the DtxR family, allowing the accumulation of the
necessary levels of this essential metal ion before shutting down the uptake machinery. In contrast, it may be that when *B. subtilis* is faced with Cd$^{2+}$ in its surroundings, MntR functions more like a member of the MerR/ArsR family of proteins, responding with great sensitivity to presence of the toxic metal ion and suppressing the corresponding uptake genes.

The question remains, under physiological conditions how does MntR selectively respond to Mn$^{2+}$ when the protein has such a low binding affinity for this metal ion? At present, no information about the *B. subtilis* metallome is available; however, the *E. coli* metallome has been determined (51, 55) and can be used to make a comparative analysis. In *E. coli*, the total metal concentrations in the cell is ~100 μM for Zn$^{2+}$ and Fe$^{2+}$, ~10 μM for Mn$^{2+}$ and Cu$^{2+}$, and submicromolar for Ni$^{2+}$ and Co$^{2+}$ (55). However, the concentrations of ‘free’ metal ions (those not bound to proteins or other biological ligands) are likely less than the total ion concentrations listed. For example, in the case of Cu$^{2+}$ and Zn$^{2+}$ the free concentration of these ions is estimated to be less than one free metal ion per cell (~10$^{-9}$ M) (49, 51). If *B. subtilis* accumulates metal ions in a similar fashion, the amounts of total Co$^{2+}$ and Ni$^{2+}$ and free Cu$^{2+}$ and Zn$^{2+}$ would be well below the binding affinity of the MntR, rendering it non-responsive to these metals (although the binding constant for Cu$^{2+}$ and MntR is not available, it would be quite surprising for MntR to demonstrate sufficiently tight binding to Cu$^{2+}$ to overcome the extremely low concentrations of this metal ion). ‘Loosely bound’ Fe$^{2+}$ concentrations in *E. coli* have been estimated at ~10 μM (56), and although we do not have quantitative binding data for MntR with Fe$^{2+}$, these concentrations could potentially compete with Mn$^{2+}$. Indeed, some mutants of MntR can be activated by Fe$^{2+}$
in vivo (35). Although no direct measurement of free Mn\(^{2+}\) is available for \textit{E. coli} or \textit{B. subtilis}, evidence suggests that these microorganisms might be more tolerant to higher cellular concentrations of Mn\(^{2+}\) relative to other transition metal ions. Toxicity tests with \textit{E. coli} grown on agar plates containing increasing concentrations of transition metal ions showed that the minimal inhibitory concentration for Mn\(^{2+}\) was 20 mM, approximate 20 times higher than that found for Co\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\), and Zn\(^{2+}\) (1 mM) and 40 times higher than that of Cd\(^{2+}\) (0.5 mM) (57). Furthermore, in several \textit{Bacillus} species, the minimal Mn\(^{2+}\) concentration required for normal vegetative growth is \(\sim 10^{-7}\) M, \(10^{-6}-10^{-3}\) M for production of secondary metabolites, and \(10^{-4}-10^{-3}\) M for cultural longevity (58). Based on these studies, one could conclude that Mn\(^{2+}\) may indeed be the only metal ion for which MntR has sufficient affinity, and is present in suitable free concentrations within the cell, which would bind to and regulate MntR during normal cellular homeostasis. Clearly, the full metallome and concentrations of free metal ions in \textit{B. subtilis} must be determined to validate this hypothesis.
CONCLUSIONS

We have determined the affinity of Mag-fura-2 for several transition metal ions, which fall in nM to μM range, making this dye useful for competition experiments with the metalloregulatory protein MntR. Evaluation of metal binding by MntR shows this protein belongs to a class of metalloregulatory proteins that possess a weak affinity for its cognate metal ion, while still eliciting tight DNA binding upon complete metal loading. EPR studies have provided evidence for a weakly coupled, binuclear metal center consistent with the revised crystallographic report of MntR ([14]). While additional structural studies will be necessary for elucidating the complete mechanisms of metal activation, the results presented here unambiguously show that the selective metal ion response of MntR does not correlate with metal binding affinities. Furthermore, the high binding affinity of MntR for Cd^{2+} versus Mn^{2+} suggests a dual role for this repressor in vivo – to maintain essential levels of Mn^{2+}, while excluding toxic Cd^{2+} with exquisite sensitivity. Last, we have demonstrated that with the exception of Cd^{2+}-MntR E99C, single site mutations do not appear to significantly alter the metal binding affinities of MntR for the metal ions tested. Interestingly, while the metal affinities appear unperturbed, the DNA-binding affinities of mutant proteins (see chapter 2 of this thesis) vary substantially. This observation suggests that the metal ions may be bound in different coordination geometry in the MntR E99C and MntR D8M proteins compared to wild type MntR. Further structural work is necessary to fully resolve the how the metal-binding affects the DNA-binding affinities of this protein.
MATERIALS AND METHODS

General

All buffers were prepared using water purified by a Labconoco Water Pro Plus purification system. All biochemical reagents were obtained from commercial suppliers and were used as provided unless otherwise specified. 8-Anilino-1-naphthalenesulfonic acid (ANS) was acquired from Sigma-Aldrich and stock solutions were prepared as previously described (17). MnCl$_2$·4H$_2$O, CoCl$_2$·H$_2$O, CdCl$_2$·H$_2$O, NiCl$_2$·6H$_2$O, and ZnCl$_2$ (99.99+%) were obtained from Aldrich. Protein purification was carried out as previously described (11, 17). The metal content/purity of all ultrapure water, buffers, protein preparations, and metal titrant solutions were determined as previously described (11) using a Perkin-Elmer Optima 3000 DV inductively coupled plasma optical emission spectrometer (ICP-OES) located at the Analytical Facility at the Scripps Institute of Oceanography. All fluorescence intensity and anisotropy experiments were performed on a Perkin-Elmer LS-55 luminescence spectrometer using a thermally jacketed cell holder that was maintained at 25 °C. All absorption spectra were collected on a Perkin-Elmer Lambda 25 spectrophotometer at ambient temperature.

Fura-2 and Mag-fura-2 were purchased from Invitrogen; stock solutions were prepared by resuspending the lyophilized powders in 1 mL of degassed ultrapure water. Dye concentrations were determined by absorption spectroscopy by using the reported extinction coefficients (28000 M$^{-1}$cm$^{-1}$ at 363 nm for Fura-2 and 22000 M$^{-1}$cm$^{-1}$ at 369 nm for Mag-fura-2). Experiments with these fluorimetric dyes were carried out in
either metal binding buffer (10 mM HEPES, pH 7.2, 100 mM KCl) or protein storage buffer (20 mM HEPES, pH 7.2@4 °C, 200 mM NaCl, 5% glycerol).

**ANS Experiments**

In an 800 μL microcuvette, a 400 μL solution containing 200 μM ANS dye and 2 μM MntR in protein storage buffer was prepared. Metal stock solutions were titrated (from 0.001 to 1000 μM) into the ANS/MntR mixture (17). The fluorescence emission of ANS was monitored and the intensity at 507 nm was plotted as a function of metal ion concentration. The data were fit with a 1:1 binding isotherm by using least-squares regression analysis software (KaleidaGraph, Synergy Software) to determine the relative affinities for Mn\(^{2+}\), Co\(^{2+}\), and Cd\(^{2+}\).

**Protein Competition with Fura-2**

In an 800 μL microcuvette, a 400 μL solution containing 5 μM Fura-2 and 50 μM MntR in protein storage buffer was prepared; the solution was incubated for 20 min at ambient temperature. To the equilibrated solution was added 5 μM metal ion (Mn\(^{2+}\), Co\(^{2+}\), or Cd\(^{2+}\)) and the resulting absorption spectra were recorded for ~16 h in 20 min intervals. The resulting spectra were compared to the spectrum just prior to addition of protein. A control experiment where 200-700 μM EDTA was added to the microcuvette (in lieu of MntR) was used to obtain the apo Fura-2 spectrum.
Metal-Binding Affinity/Stoichiometry of Mag-fura-2

For all fluorescence experiments employing the dye Mag-fura-2 the fluorescence excitation was scanned from 250-450 nm while monitoring emission at 505 nm. Excitation and emission slits were set at 10 and 5 nm, respectively. To determine the metal-binding stoichiometry of Mag-fura-2 with Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$ electronic absorption spectroscopy was used in combination with the method of continuous variation (Job’s method) (59). In an 800 μL microcuvette, a 400 μL solution containing Mag-fura-2 and the divalent metal ion of interest was prepared for a combined total concentration of 42 or 66 μM in metal binding buffer. Nine solutions were prepared for each metal ion, with the mole fraction of Mag-fura-2 ranging from 0 to 1. Each solution was allowed to equilibrate at ambient temperature for 5 min before recording the absorbance spectrum from 200-500 nm. The absorbance of Mag-fura-2 alone was subtracted from each experimental spectrum and the data was analyzed by plotting $A_{369}$ vs. mole fraction of Mag-fura-2 ($X_{Mf-2}$).

To determine the metal-binding affinity of Mag-fura-2 with Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$ ions two sets of experiments were carried out, one in metal binding buffer and a second in protein storage buffer. In a 4 mL cuvette, a 2 mL solution containing 0.5 μM Mag-fura-2 and 1 or 10 mM metal ion was prepared; the solution was allowed to equilibrate at ambient temperature for 3 min. A competing metal ion was then titrated (0 to ~5 mM, <10% total volume change) into the preincubated mixture and the fluorescence excitation spectra were recorded. A 3 min equilibration time was allowed between additions of competing metal ion. In experiments designed to determine the affinity of Mag-fura-2 for either Co$^{2+}$, Mn$^{2+}$, or Ni$^{2+}$ (binding of these
metal ions quenches the Mag-fura-2 fluorescence, as previously observed for Fura-2) 
(19), Ca\(^{2+}\) was used as the preincubation metal (\(K_d = 25 \mu\text{M}\), as determined by the 
manufacturer, Invitrogen) (23). In experiments designed to determine the affinity of 
Mag-fura-2 for Zn\(^{2+}\) or Cd\(^{2+}\) (binding of these metals generates a fluorescence 
excitation spectrum with a maximal \(\lambda_{ex} \sim 330 \text{ nm}\)) the preincubation metal was Co\(^{2+}\), 
Mn\(^{2+}\), or Ni\(^{2+}\). The reported Mag-fura-2 \(K_d\) of 25 \(\mu\text{M}\) for Ca\(^{2+}\) was used as the reference 
for experiments performed in either metal binding or protein storage buffer. It has been 
reported that Mag-fura-2 metal binding affinities do not change significantly in the pH 
range of 6.4-7.5 and within ionic strengths 0.1-0.2 M (43, 60); therefore the \(K_d\) of 25 
\(\mu\text{M}\) should be satisfactory under both buffer conditions used here. The fluorescence 
intensity at 505 nm when excited with an excitation wavelength of \(\lambda_{ex}=330 \text{ nm}\) was fit 
using a custom DYNAFIT script that describes the competition between two metal ions 
for the dye (see Addendum following “Materials and Methods”); reported \(K_d\) values are 
an average of at least three individual experiments. The program DYNAFIT does not 
utilize a single explicit equation (binding isotherm expression) to fit thermodynamic 
data, but fits the data employing simultaneous nonlinear algebraic equations (27, 28).

**Protein Competition with Mag-fura-2**

In an 800 \(\mu\text{L}\) microcuvette, a 400 \(\mu\text{L}\) solution containing 0.5 \(\mu\text{M}\) Mag-fura-2 
and MntR (10-100 \(\mu\text{M}\)) was prepared in protein storage buffer. The mixture was 
incubated at ambient temperature for 3 min. The metal ion of interest was titrated into 
the preincubated solution with a 3 min equilibration time between additions. 
Fluorescence excitation spectra were recorded from 250-450 nm at each point in the
titration. The fluorescence intensity at 505 nm with an excitation wavelength of \(\lambda_{\text{ex}}=330\) nm (Zn\(^{2+}\), Cd\(^{2+}\)) or \(\lambda_{\text{ex}}=380\) nm (Co\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\)) was fit using a custom DYNAFIT scripts that describe the competition between the dye and the protein for the metal ion (see Addendum following “Materials and Methods”). Reported \(K_d\) values are an average of at least three individual experiments.

Using absorption spectroscopy an analogous competition experiment was also carried out. In an 800 \(\mu\)L microcuvette, a 400 \(\mu\)L solution containing 6.5 \(\mu\)M Magfura-2 and 50 \(\mu\)M MntR was prepared in protein storage buffer. The mixture was incubated at ambient temperature for 3 min. A Co\(^{2+}\) solution was titrated from 0.03 to 200 \(\mu\)M into the preincubated solution with a 3 min equilibration time between additions. Absorbance spectra were recorded from 250-500 nm and binding of Co\(^{2+}\) to the dye was monitored by disappearance of the apo Magfura-2 absorbance at \(\sim362\) nm. Data were modeled using a custom DYNAFIT (27) script that fit competition between the dye and the protein for the metal ion (see Addendum following “Materials and Methods”); reported \(K_d\) values are an average of at least three individual experiments.

**Alternative fitting procedure for fluorescence competition titration data.**

Alternatively, the competition experiment data can be fit by adapting a series of binding isotherm equations used for competition absorption spectrophotometry (59, 61, 62). Fitting of the data to a single metal binding site by the latter procedure gives values consistent with those obtained from the more explicit DYNAFIT procedure. Dissociation constants could be obtained by the method described by Connors (see ref.
Application of these equations have appeared in the work of Imperiali (62) and others.

The variable \( Q \) is defined as the ratio of free to metal-bound Mag-fura-2. The variable \( P \) is the concentration of the MntR-metal complex. Under our experimental conditions, these variables can be expressed as follows:

\[
Q = \frac{e - e_{LL}}{e_f - e} \quad (3.1)
\]
\[
P = L_T - \frac{1}{Q K_I} - \frac{I_T}{Q+1} \quad (3.2)
\]

Where \( e, e_f, \) and \( e_{LL} \) are the observed, free, and metal-bound fluorescence intensities of Mag-fura-2. \( L_T \) is the total metal concentration, \( I_T \) is the total Mag-fura-2 concentration, and \( K_I \) is the association constant between Mag-fura-2 and the metal ion (Table 3.1).

The variables \( Q \) and \( P \) can be related by the following expression:

\[
\frac{S_T}{P} = \frac{K_I}{K_{11}} Q + 1 \quad (3.3)
\]

where \( S_T \) is the total MntR concentration and \( K_{11} \) is the binding constant of interest (between MntR and the metal ion). A plot of \( S_T/P \) versus \( Q \) gives a straight line, the slope of which can be used to determine \( K_{11} \). Example plot is given in Figure 3.18 for \( \text{Co}^{2+} \) analyzed in this manner. The small number of data points used is due to the
limitation that the usable values of $Q$ are between 0.3-3 (see ref. (59), page 180); nevertheless, the values are in good agreement with those generated by DYNAFIT.

![Graph showing linear relationship between $S_{IP}$ and $Q$](image)

**Figure 3.18.** Fit of competition titration between Mag-fura-2 and MntR for Co$^{2+}$ using the method described by Connors (see equation 3.3). The dissociation constant obtained from the slope of the line (using the binding constants from Table 3.1) is 4.7 μM, in good agreement with the values calculated by the program DYNAFIT (Table 3.2).

**Fluorescence Anisotropy**

Fluorescence anisotropy experiments were performed as previously described (11). In a 4 mL cuvette were placed 10 nM dsmntH26 (fluorescein-labeled strand of dsmntH26 = 5'-6F-GAATAATTTGCCCTAAGGAAACTCTC-3') (11) and 10, 100, or 1000 μM (final concentration) of divalent metal ion (Mn$^{2+}$, Co$^{2+}$, or Cd$^{2+}$). To this solution was added increasing amounts of MntR (0-1.5 μM). Measurements were collected with an excitation wavelength of 492 nm (slit width 15 nm), an emission
wavelength of 520 nm (slit width 20 nm), and a 1.0 s integration time. The g-factor for all of the experiments was 1.15±0.08. The $r_{\text{obs}}$ versus $[\text{MntR}]_{\text{total}}$ data were fit to a 1:1 binding isotherm model (non-dissociable dimer) (II) by using least-squares regression analysis software (KaleidaGraph, Synergy Software).

**EPR Spectroscopy**

X-band (9 GHz) EPR spectra were recorded on a Bruker ESP 300 spectrometer equipped with an Oxford ESR 910 cryostat for low-temperature measurements. The microwave frequency was calibrated with a frequency counter, and the magnetic field was calibrated with a NMR gaussmeter. The temperature was calibrated with resistors (CGR-1-1000) from LakeShore. A modulation frequency of 100 kHz was used for all EPR spectra. All experimental data were collected under nonsaturating microwave conditions. Room temperature measurements used a flat quartz cell positioned in an E-field null plane of the microwave cavity.

EPR spectra were analyzed by diagonalization of the spin Hamiltonian $H = -2J\mathbf{S}_1\cdot\mathbf{S}_2 + H_{\text{dipolar}} + H_1 + H_2$, where $J$ is the isotropic exchange coupling between metal sites, $H_{\text{dipolar}}$ is the through space Mn-Mn magnetic dipolar interaction, and $H_1$ and $H_2$ contain the Zeeman and zero-field terms for the individual Mn$^{2+}$ ions (32). The simulations were generated with consideration of all intensity factors relative to a spin standard (CuEDTA), which allowed computation of simulated spectra for a specific sample concentration. The simulations therefore allow a quantitative determination of protein signal intensities. The Windows software package SpinCount was used and is
available for general application to any mono- or binuclear metal complex by contacting Prof. Mike Hendrich, hendrich@andrew.cmu.edu.

For single value decomposition (SVD) of data (63), the spectra were arranged into an N rows by M columns matrix, A, with each column representing a spectrum with N data points. This matrix can be expressed in terms of three matrices U, S, and V such that \( A = U S V^T \). In this representation, S is an \( N \times N \) diagonal matrix of non-negative elements containing the singular values of matrix A. The diagonal elements of the matrix \( S^2 \) are the eigenvalues, and the columns of V are the corresponding eigenvectors of \( A^T A \). Within the context of a physical model, the entries in V can be related to the amplitudes of the base spectra for each of the titration points. The physical model in our particular application is the sequential binding two metal sites to the protein,

\[
E + Mn \xrightarrow{K_1} EMn + Mn \xrightarrow{K_2} EMn_2; K_1 = \frac{[EMn]}{[E][Mn]}; K_2 = \frac{[EMn_2]}{[EMn][Mn]} \quad (3.4)
\]

\[
[E_T] = [E] + [EMn] + [EMn_2], \quad [Mn_T] = [Mn] + [EMn] + 2[EMn_2],
\]

where Mn, E, EMn, and EMn_2 represent the concentrations of Mn(H_2O)_6^{2+}, and apo-, mono-, and di-metal bound protein species, respectively. The total concentrations of protein and metal are \( E_T \) and \( Mn_T \), and \( K_1 \) are the equilibrium constants. From equation 3.4, the concentrations of Mn, EMn, and EMn_2 can be determined in terms of the binding constants \( K_1 \) and \( K_2 \) (30). In the limit \( K_1 \ll K_2 \), the low concentration of the EMn species will render it undetectable. Thus, the mononuclear binding step is not measurable, and the fit the data will be primarily sensitive to the product \( K_1K_2 \). The
experimental matrix $V$ is calculated from $A$ using a standard SVD routine \((64)\). A computer program was constructed which then least-square fits the value of the product $K_1K_2$ to give the best match of the theoretical matrix $CP$ constructed from equation 3.4 to the experimental matrix $V$. $C$ is an $n \times r$ matrix containing the species concentrations as a function of total added metal concentration (for our application, $r = 2$ species, $n = 7$ titration points), and $P$ is a set of linear parameters \((63)\).

EPR samples were prepared by titrating $\text{Mn}^{2+}$ into a solution of apoMntR. Stock solutions of $\text{MnCl}_2$ (Aldrich) were prepared daily in protein buffer with doubly distilled water. Quantitation of $\text{Mn}^{2+}$ stock solutions were determined through double integration of the EPR spectra, and compared against atomic absorption standards for $\text{Mn}^{2+}$ (Aldrich). For each metal addition, MntR was titrated with the appropriate amount of stock solution in successive additions. For room temperature measurements, after each addition the sample was incubated for ten minutes at $4 ^\circ \text{C}$ prior to recording the EPR spectrum. For low temperature measurements, each sample was incubated for ten minutes at $4 ^\circ \text{C}$ and then frozen in liquid $\text{N}_2$, followed by recording of the spectrum. Unless noted, the buffer for all samples was 20 mM HEPES (Sigma), pH 7.2, 300 mM NaCl, 5% glycerol.

The text of chapter 3, in full, is a reprint of the material as it appears in the journal Biochemistry, 2006, vol. 45(51):15359-15372 “Metal Binding Studies and EPR Spectroscopy of the Manganese Transport Regulator MntR”. The dissertation author was the primary researcher and/or author and the co-authors (Gunderson WA, Hendrich
MP, Cohen SM) listed in this publication contributed to or supervised the research which forms the basis for this chapter. The authors thank Dr. Emmanuel Guedon and Prof. John D. Helmann (Cornell University) for providing the protein vectors, Prof. Arthur Glasfeld (Reed College) for many helpful discussions and disclosure of unpublished data, and Dr. Annette Deyhle (Scripps Institute of Oceanography) for access and assistance with the ICP-OES. We thank Dr. Petr Kuzmic for extensive discussions on the use of DYNAFIT, suggested improvements to experimental design, and assistance with the data fitting analysis.
**Mag-fura-2 Only Titration:** This script was used to fit the competition of two metal ions for the dye Mag-Fura-2 and thereby measure the affinity of Mag-Fura-2 for a metal ion that forms a fluorescent complex with the dye. Specifically, Mag-Fura-2 "MF2" is incubated with a metal that quenches its fluorescence "A" and then titrated with a second metal "B" that results in a fluorescent metal-dye complex "BMF2".

```plaintext
;===================================================================
[task]
task = fit
data = equilibria

[mechanism]
A + MF2 <=> AMP2 : Kd1 dissoc
B + MF2 <=> BMF2 : Kd2 dissoc

[constants]
Kd1 = .93
Kd2 = .02 ?

[concentrations]
MF2 = 0.5
A   = 10000

[responses]
MF2  = 100 ?
BMF2 = 450 ?

[equilibria]
directory ./Data/directory_name
extension txt
offset auto ?

variable B
file file_name_here

[output]
directory ./output/directory_name/file_output_name

[end]
```
Mag-fura-2 vs. MntR (1 binding site) Titration: This script was used to fit the competition of Mag-Fura-2 and MntR for a metal ion that forms a 1:1 complex with the MntR monomer. Specifically, Mag-Fura-2 is incubated with MntR and M is titrated into that solution; formation of the metal-Mag-Fura-2 complex “MMF2” results in one of two possibilities:

1) with Zn and Cd, complex formation is accompanied by formation of a new peak in the fluorescence excitation spectrum, or
2) with Mn, Co, and Ni, complex formation is accompanied by quenching of the dye signal in the fluorescence excitation spectrum.

;=====================================================================
[task]
task = fit

data = equilibria

[mechanism]
M + MF2 <=> MMF2 : Kd1 dissoc
M + MntR <=> MMntR : Kd2 dissoc

[constants]
Kd1 = .104
Kd2 = .001

[concentrations]
MF2 = 0.5
MntR = 10.0

[responses]
MF2 = 100
MMF2 = 450

[equilibria]
directory ./Data/directory_name
extension txt
offset auto
variable M
file file_name_here

[output]
directory ./output/directory_name/file_output_name

[end]
DYNAFIT MATRIX OUTPUT

Reaction Mechanism
The chemical system analyzed in here is described by the following reaction mechanism:

\[
\begin{align*}
\text{CdMF}_2 & \rightleftharpoons \text{Cd} + \text{MF}_2 & K_{d1} \\
\text{CdMntR} & \rightleftharpoons \text{Cd} + \text{MntR} & K_{d2}
\end{align*}
\]

MF\(_2\) = Mag-fura-2
The mathematical model is a system of simultaneous nonlinear algebraic equations described in I & Nancollas (1972). Species that participate in equilibrium steps are listed in the formula matrix \( F \) below. Stability constants of complexes are computed from the reaction equilibrium constants according to the stability constant matrix \( B \).

**Formula matrix \( F \)**

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<tr>
<th></th>
<th>CdMF(_2)</th>
<th>CdMntR</th>
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<tbody>
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</tr>
<tr>
<td>MF(_2)</td>
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<td>0</td>
</tr>
<tr>
<td>MntR</td>
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<td>1</td>
</tr>
</tbody>
</table>

**Stability constant matrix \( B \)**

<table>
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<th></th>
<th>CdMF(_2)</th>
<th>CdMntR</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{d1} )</td>
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<td>0</td>
</tr>
<tr>
<td>( K_{d2} )</td>
<td>0</td>
<td>-1</td>
</tr>
</tbody>
</table>
Mag-fura-2 vs. MntR (2 independent binding sites) Titration: This script was used to fit the competition of Mag-Fura-2 and MntR for Cd ions in a model where a 2:1 complex with the MntR monomer is formed. Specifically, Mag-Fura-2 is incubated with a MntR and Cd is titrated into that solution; formation of the Cd-Mag-Fura-2 complex is accompanied by a new peak in the fluorescence excitation spectrum of Mag-Fura-2.

;=====================================================================

[task]
task = fit
data = equilibria

[mechanism]
Cd + MF2    <==> CdMF2      :    Kd1    dissoc
Cd + MntR   <==> CdMntR     :    Kd2    dissoc
Cd + CdMntR <==> CdCdMntR   :    Kd3    dissoc

[constants]
Kd1 = .104
Kd2 = .00001 ?
Kd3 = .001 ?

[concentrations]
MF2   =  0.5
MntR  = 10.0

[responses]
MF2 =   100 ?
CdMF2 = 450 ?

[equilibria]
directory ./Data/directory_name
	extension txt

offset auto ?

variable Cd

file file_name_here

[output]
directory ./output/directory_name/file_output_name

[end]
DYNAFIT MATRIX OUTPUT

Reaction Mechanism
The chemical system analyzed in here is described by the following reaction mechanism:

\[
\begin{array}{ccc}
\text{CdMF}_2 & \rightleftharpoons & \text{Cd} + \text{MF}_2 & K_{d1} \\
\text{CdMntR} & \rightleftharpoons & \text{Cd} + \text{MntR} & K_{d2} \\
\text{CdCdMntR} & \rightleftharpoons & \text{Cd} + \text{CdMntR} & K_{d3}
\end{array}
\]

$\text{MF}_2 = \text{Mag-fura-2}$

The mathematical model is a system of simultaneous nonlinear algebraic equations described in I & Nancollas (1972). Species that participate in equilibrium steps are listed in the formula matrix $F$ below. Stability constants of complexes are computed from the reaction equilibrium constants according to the stability constant matrix $B$.

**Formula matrix F**

<table>
<thead>
<tr>
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<th>CdMF$_2$</th>
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<th>CdCdMntR</th>
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</tr>
<tr>
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**Stability constant matrix B**

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<th>CdMntR</th>
<th>CdCdMntR</th>
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<tbody>
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<td>0</td>
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<tr>
<td>$K_{d2}$</td>
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<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>$K_{d3}$</td>
<td>0</td>
<td>0</td>
<td>-1</td>
</tr>
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</table>
REFERENCES


(48) Busenlehner, L. S., Weng, T.-C., Penner-Hahn, J. E., and Giedroc, D. P. (2002) Elucidation of primary ($\alpha_3N$) and vestigial ($\alpha_5$) heavy metal-binding sites in *Staphylococcus aureus* pI258 CadC: evolutionary implications for metal ion selectivity of ArsR/SmtB metal sensor proteins. *J. Mol. Biol.* 319, 685-701.


Chapter 4.

Conformational studies of MntR using deuterium exchange mass spectrometry (DXMS)
ABSTRACT

Having characterized the metal binding affinities of MntR and the DNA-binding activation profiles for the relevant metal ions, the focus of the current study is to investigate structural changes of MntR in solution upon binding divalent transition metal ions. Deuterium exchange mass spectrometry (DXMS) was utilized to investigate the deuterium exchange dynamics between apo-, Co$^{2+}$-, Cd$^{2+}$-, and Mn$^{2+}$- MntR. Comparing the rates of deuteration of each metal-bound form of MntR reveals that the N-terminal DNA-binding motif is more mobile in solution than the C-terminal dimerization domain. Furthermore, significant protection from deuterium exchange is observed in the helices that contribute metal-chelating amino acids in the metal binding site of MntR. Interestingly, the bulk of the DNA-binding winged helix-turn-helix motif shows no difference in deuterium exchange upon metal binding. Mapping of the deuteration patterns onto the crystal structures of MntR yields insight into how metal binding affects the protein structure. Metal binding acts to rigidify helix 4 of MntR, thereby limiting the mobility of the protein and reducing the entropic cost of associated with DNA binding.
INTRODUCTION

The DNA-binding affinities in presence of various metal ions determined in chapter 2 and the metal binding affinities of MntR determined in chapter 3 beg the question of how is MntR able to translate the metal-binding event into the DNA-binding ability of this protein. To fully characterize MntR and to address this discrepancy in DNA-binding activation, we resolved to determine the metal-mediated mechanism of DNA binding. Prior work from our laboratory (chapter 2 of this thesis) has focused on biophysical characterization of MntR by using circular dichroism (CD) spectroscopy to investigate the secondary structure of this protein, thermal melting (also monitored with CD spectroscopy) to examine protein stability, and metal titration experiments in the presence of hydrophobic dyes to examine changes in tertiary structure upon metal binding (1, 2). These experiments revealed that metal-binding does not affect the secondary structure of MntR (which is largely $\alpha$-helical), but does affect either the tertiary or quaternary structure of MntR. While the formation of the binuclear metal site of MntR has been implicated as an essential step for the mechanism of MntR activation, the structural changes that occur upon metal binding and the complete mechanism of action have not been conclusively determined. Herein we employ deuterium exchange mass spectrometry (DXMS) as a means to examine this mechanism.

Deuterium exchange has been used in biological NMR experiments and recently has become more prominent in conjunction with mass spectrometry (3). Deuterium exchange relies on the ability of hydrogen atoms in the protein to exchange with
deuterons from the solvent (4, 5). The rate with which the exchange occurs is dependent on the solvent exposure of each residue, a property which further depends on how dynamic the residues are in solution (4, 5). The exchange of hydrogen atoms on the amino acid side chains occurs on a rapid timescale that is difficult to monitor by spectroscopic methods; however, the amide hydrogen atoms of the protein backbone exchange more slowly, which permits monitoring of deuterium incorporation by NMR and MS methods (4, 5). A typical time-course experiment for DXMS is illustrated in Figure 4.1.

Regions of the protein affected by cofactor binding will exhibit different deuterium exchange patterns compared to apo-protein. In particular, molecular weight analysis of the protein fragments following the DXMS protocol yields insight into whether cofactor binding protects or exposes the protein to deuterium exchange; however, the resolution of such experiments is restricted to the digested protein fragments, and it is generally not possible to localize deuterium atoms to individual amino acids using DXMS. Furthermore, in contrast to X-ray crystallography and NMR spectroscopy, absolute positions of the elements of protein structure relative to each other cannot be determined with DXMS. Nevertheless, because the rate of the exchange of backbone amide hydrogen atoms depends on their exposure to solvent, the incorporation of deuterium can be used to report on various aspects of protein structure and dynamics such as folding and stability, ligand binding, aggregation, and protein-protein interactions [12, 14-28].
Figure 4.1. Schematic diagram for DXMS. Protein is diluted into deuterated buffer, thus initiating deuterium exchange with amide hydrogen atoms in the protein backbone ("on-exchange"); at selected time points, samples are removed and quenched at low temperature and pH to limit further exchange. For DXMS protocol, samples are digested with pepsin protease and resulting fragments are HPLC separated prior to MS analysis. Subsequent analysis of data yields information on deuterium location and quantity.

DXMS has been utilized to investigate metal-induced structural changes of proteins by comparing apo and holo forms (6, 7). For example, de Peredo et al investigated Mn$^{2+}$ binding to the metalloregulatory protein Fur, a functional homologue of DtxR found in gram-negative bacteria (6). Digestion of deuterated apo, holo, and DNA-bound holo-Fur, followed by mass spectrometry showed that certain regions of the protein were more deuterated upon metal binding compared to apo-Fur, implying
that metal binding results in a structural change that exposes these residues to deuterium exchange. However, upon DNA binding the protein again exhibits exchange similar to apo-protein. This information suggested that Fur takes on a more ‘open’ conformation upon metal binding, thus exposing the DNA-binding helix-turn-helix (HTH) motif to facilitate Fur-DNA interactions (6).

Three recent reports in the literature describe the crystallographic structures of MntR reconstituted with several metal ions including, Mn$^{2+}$, Cd$^{2+}$, Zn$^{2+}$, and Ca$^{2+}$ and most recently, the structure of apo-MntR (8-10). The authors suggest that the protein can be described by two domains. The N-terminal domain (residues 1-70, $\alpha$-helices 1-3 and $\beta$-strands 1-2) contains a winged HTH motif involved in DNA-binding, while the C-terminal dimerization domain (residues 70-142, $\alpha$-helices 4-7) contains the dimerization interface (Figure 4.2). The overall conformations of MntR in the various metal bound forms are almost identical, with only minor structural variations between the metals sites and the mobile region (residues 54-58) linking the two beta strands of various isoforms (Figures 3.1 and 3.2) (9). In terms of the metal binding sites, the Mn$^{2+}$, Cd$^{2+}$, and Ca$^{2+}$-loaded structures exhibit binuclear metal active sites with similar coordination environments. In the binuclear metal site, Glu11, His77, Glu99, Glu102, and up to 2 water molecules bind in site A and Asp8, Glu11, Glu102, His103, and 1 water molecule bind in site C, recruiting these ligands from helices 1, 4, and 5. The Zn$^{2+}$-bound form of MntR is mononuclear with the Zn$^{2+}$ ion bound in site A in a tetrahedral geometry (Figure 3.2) (9).
Figure 4.2. Structure of Mn$^{2+}$-MntR (PDB ID: 2F5F) with Mn$^{2+}$ ions shown as blue spheres with the ‘A’ and ‘C’ sites labeled in one monomer. One monomer unit is shown in gray, while the other monomer is shown in a gradient map coloring from N-terminus (green) to C-terminus (red). Metal-binding amino acids are highlighted in magenta.

While several MntR crystal structures have been determined (8, 9), the effects of metal coordination on the solution structure of this protein have not been described until recently (10). The recent structure of apo-MntR shows an overall conformation quite similar to the previously determined holo-forms; however, the apo structures did show significant interdomain movement. Based on these movements, the authors proposed that MntR undergoes a disorder to order transition upon metal binding, which thereby mediates DNA binding (10). In light of these findings, it is of interest to determine
whether more structural dynamics are present in solution and how such dynamics play a role in the mechanism of MntR. Herein, DXMS is used to compare the solution structure of apo- and holo-MntR in order to identify regions affected by M$^{2+}$-binding and to elucidate how the formation of the binuclear metal site contributes to the mechanism of metal-mediated activation for DNA binding of this protein. In addition, comparison of exchange rates between apo, Mn$^{2+}$, Cd$^{2+}$, and Co$^{2+}$-bound forms of MntR are used to reveal the solution dynamics of MntR.
RESULTS

In order to analyze results from the time-course experiments, it is crucial first to determine which peptide fragments are generated upon protease digestion of MntR. Use of GuHCl in the quench buffer results in unfolding, which exposes more of the protein, thereby improving digestion by proteases and increasing the number of potential fragments that can be sampled from the deuterium exchange experiment. High GuHCl concentrations were employed to ascertain that both the apo- and holo-MntR would be equally unfolded under the same conditions, while the low pH and temperature under which digestion was performed limited the back-exchange of the incorporated deuterium ions into the bulk solution. A fragmentation map of apo-MntR showed thorough coverage of the protein, with 64 peptide fragments (33 unique fragments, with 31 redundant fragments that carry different charge states) available for monitoring the exchange experiment (Figure 4.3). The number of deuterium atoms incorporated per fragment was monitored for 12 unique, non-overlapping peptide fragments and 2 non-unique peptide fragments (Table 4.1 and Figures 4.4 - 4.7). These fragments were then used to construct plots of percent deuteration described below.
Table 4.1. Fragments of MntR monitored during the time-course experiment. The monoisotopic m/z is the calculated mass, while the ‘centroid’ is the geometric centroid of isotopic envelopes (11). Residues shaded in gray are not included for calculating MaxD, as these amino acids do not retain deuterium (see “Materials and Methods” section). Fragments 93-XX and 102-111 overlap with 93-111. Fragment 93-XX varied between apo and holo forms of MntR.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>2° Structure</th>
<th>Possible Function</th>
<th>Amino Acid Sequence</th>
<th>monoisotopic m/z</th>
<th>centroid m/z</th>
<th>z</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-8</td>
<td>α.1</td>
<td>Metal-Binding</td>
<td>MTTPSMD</td>
<td>910.34</td>
<td>911.809</td>
<td>1+</td>
</tr>
<tr>
<td>11-15</td>
<td>α.1</td>
<td>Metal-Binding</td>
<td>YIEQIYM</td>
<td>958.45</td>
<td>960.046</td>
<td>1+</td>
</tr>
<tr>
<td>18-27</td>
<td>Largely unstructured</td>
<td>DNA binding (winged HTH)</td>
<td>LIEEKGYARVSD</td>
<td>1378.71</td>
<td>1380.487</td>
<td>1+</td>
</tr>
<tr>
<td>30-32</td>
<td>α.2</td>
<td>DNA binding (winged HTH)</td>
<td>IAEL</td>
<td>515.3</td>
<td>516.641</td>
<td>1+</td>
</tr>
<tr>
<td>33-42</td>
<td>α.3; some unstructured</td>
<td>DNA binding (winged HTH)</td>
<td>ALAVHPSSVTKM</td>
<td>1055.54</td>
<td>1057.194</td>
<td>1+</td>
</tr>
<tr>
<td>45-52</td>
<td>α.3; some unstructured</td>
<td>DNA binding (winged HTH)</td>
<td>VQKLDKDEYL</td>
<td>1249.66</td>
<td>1251.292</td>
<td>1+</td>
</tr>
<tr>
<td>54-62</td>
<td>β1 and β2; some unstructured</td>
<td>DNA binding (winged HTH)</td>
<td>LIYEKYRGLVL</td>
<td>1365.8</td>
<td>1367.533</td>
<td>1+</td>
</tr>
<tr>
<td>65-80</td>
<td>α.4</td>
<td>Metal-Binding</td>
<td>TSKGGKIKGRLVYRHELL</td>
<td>2125.28</td>
<td>1064.254</td>
<td>2+</td>
</tr>
<tr>
<td>88-91</td>
<td>α.5; some unstructured</td>
<td>Dimerization Interface</td>
<td>LRIIGVDE</td>
<td>913.52</td>
<td>914.961</td>
<td>1+</td>
</tr>
<tr>
<td>93-111</td>
<td>α.5 and α.6; some unstructured</td>
<td>Dimerization Interface</td>
<td>EEKIYNDVGEIHHLSWNSID</td>
<td>2526.17</td>
<td>1204.914</td>
<td>2+</td>
</tr>
<tr>
<td>93-XX</td>
<td>α.5, coverage varied between isoforms</td>
<td>Dimerization Interface, Metal-binding</td>
<td>N/A (NOT APPLICABLE)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>102-111</td>
<td>α.6; some unstructured</td>
<td>Dimerization Interface, Metal-binding</td>
<td>GEIHHLSWNSID</td>
<td>1408.66</td>
<td>1408.383</td>
<td>1+</td>
</tr>
<tr>
<td>114-116</td>
<td>α.6</td>
<td>Dimerization Interface</td>
<td>RGDL</td>
<td>572.33</td>
<td>573.611</td>
<td>1+</td>
</tr>
<tr>
<td>119-142</td>
<td>α.7; some unstructured</td>
<td>Dimerization Helix</td>
<td>VQYFEEDARKKDQLKSIQKKTIEHNNQ</td>
<td>3213.62</td>
<td>1608.694</td>
<td>2+</td>
</tr>
</tbody>
</table>
Figure 4.3. Fragmentation map of apo-MntR at 25 °C. Each bar represents a unique MntR fragment; identical bars represent different charge states of the same fragment. Structural features such as α-helices and β-sheets are highlighted in yellow. Fragments used for DXMS data analysis are highlighted in gray.
Figure 4.4. Time-dependent deuteration of fragments from the N-terminus of MntR (residues 3-32). Apo (♦), Co\textsuperscript{2+} (▲), Cd\textsuperscript{2+} (▼), and Mn\textsuperscript{2+} (●).
Figure 4.5. Time-dependent deuteration of fragments from the N-terminus of MntR (residues 33-62). Apo (●), Co$^{2+}$ (▲), Cd$^{2+}$ (▼), and Mn$^{2+}$ (●).
Figure 4.6. Time-dependent deuteration of fragments from the C-terminus of MntR (residues 65-111). Apo (◇), Co\(^{2+}\) (▲), Cd\(^{2+}\) (▼), and Mn\(^{2+}\) (●).
Figure 4.7. Time-dependent deuteration of fragments from the C-terminus of MntR (residues 114-142). Apo (▲), Co²⁺ (▲), Cd²⁺ (▼), and Mn²⁺ (●).

**Deuterium Exchange on apo-MntR**

By following the 14 selected peptide fragments and analyzing the number of deuterons incorporated over time, one can develop a model for the structural dynamics of MntR in solution. In order to normalize the rates of exchange between the different regions of MntR, deuteration data is presented in terms of percent deuteration rather than the total number of deuterons incorporated into a peptide fragment. Percent deuteration is calculated as the ratio of the number of incorporated deuterons on a fragment versus the total number of exchangeable deuterons (MaxD) as described in Materials and Methods section.

On the basis of the aforementioned analysis, the data show that the N-terminal DNA-binding domain (residues 1-62) of MntR generally exchanges more rapidly than the C-terminal (residues 83-142) dimerization domain. Within the N-terminus, with the exception of residues 11-15 and 30-32, deuterium exchange is essentially complete.
(≥80%) within the first ten minutes of the reaction (Figure 4.8). The exchange on the fragment containing amino acids 3-8 is particularly rapid, with almost 80% deuteration reached after 10 seconds. In contrast, fragments 11-15 and 30-32 are very resistant to deuterium exchange, displaying less than 25% $^2$H incorporation one hour after initiation of the exchange reaction (Figure 4.8). The anti-parallel $\beta$-strands (fragment 54-62, which forms the wing of the HTH motif) deemed “mobile” in crystallographic reports (9) indeed exhibit the fastest initial exchange within the DNA-binding motifs. The link between N- and C-terminal domains, helix 4 (fragment 65-80), also undergoes rapid exchange, at a rate comparable to the N-terminus (Figure 4.9).

In contrast to the N-terminus and helix 4, the exchange kinetics of the apo-MntR are comparatively slow at the C-terminal dimerization domain, specifically at the dimer interface (residues 86-116). As was anticipated on the basis of crystallographic and biophysical evidence regarding the oligomeric state of MntR, fragments 86-91 and 114-116 are highly resistant to deuterium exchange, with residues 93-111 slightly more exposed than the rest of this region (Figure 4.9, Figure 4.11). In contrast, helix 7 (fragment 117-142) of the C-terminus undergoes rapid $^2$H exchange compared to the dimerization interface. Moreover, helix 7 exchanges at a comparable rate to that of the N-terminus of MntR, but exhibits lower total deuteration, never reaching greater than 80% deuteration even at the longest incubation times (Figure 4.9).
Figure 4.8. Percent deuteration exchange maps for MntR (1-62). Metal-binding residues are boxed. Time-course experiments with 10 s, 30 s, 1 min, 2.5 min, 10 min, 1 h, 4 h, and 24 h time points (top to bottom within each experiment).
Figure 4.9. Percent deuteration exchange maps for MntR (63-142). Metal-binding residues are boxed. Time-course experiments with 10 s, 30 s, 1 min, 2.5 min, 10 min, 1 h, 4 h, and 24 h time points (top to bottom within each experiment).
Deuterium Exchange on Co\textsuperscript{2+}-MntR

Most of the N- and C-terminal domains of Co\textsuperscript{2+}-MntR exhibit exchange that is comparable to apo-MntR (Figure 4.8, Figure 4.9). However, deuteration patterns in regions affected by metal binding exhibit differences. In particular, the N-terminal fragment 11-15 showed slower \textsuperscript{2}H incorporation, with less than 40% deuteration after four hours. In the C-terminal domain of Co\textsuperscript{2+}-MntR, fragment 65-80 is protected from initial exchange (<40% within 10 min), although over time it deuterates comparably with apo-MntR. Both peptide fragments, 11-15 and 65-80, show slower rates of deuteration when compared to apo-MntR (Figure 4.10).

**Figure 4.10.** Deuteration of fragments that possess metal-ligating residues: 11-15, Glu11 (left); 65-80, His77 (center); 93-111, Glu99, Glu102 and His103 (right). Apo (●), Co\textsuperscript{2+} (▲), Cd\textsuperscript{2+} (▼), and Mn\textsuperscript{2+} (●).

Because Co-MntR is mononuclear and occupies binding site A (A. Glasfeld, personal communication), we expected exchange dynamics to be different within fragment 93-111 when compared to apo- as well as Mn\textsuperscript{2+}/Cd\textsuperscript{2+} bound MntR. Analysis of this region
shows that apo- and Co$^{2+}$-MntR deuterate at similar rates; thus in order to further scrutinize this fragment, peptides 93-XX and 102-111 were also monitored (where ‘XX’ indicates the terminal amino acid in the fragment varied between holo forms). The advantage of analyzing 2 smaller fragments (compared to 93-111) is the increased resolution and ability to better localize the region of the exchange. We were unable to map out the same fragment on all reconstituted forms of MntR and hence cannot directly compare $^2$H exchange in this region; however, we were able to monitor similar regions between the different forms of MntR. Comparison in this region was based on fragments 93-97 for apo-MntR, 93-100 for Co$^{2+}$- and Cd$^{2+}$-MntR, and 93-99 for Mn$^{2+}$-MntR. Interestingly, this region is in close proximity to and contains some of the metal binding site residues for MntR. Specifically, the metal binding residues Glu99, Glu102, and His103 are located in this region, which may protect this site from pepsin cleavage resulting in the slightly longer fragments observed. Because the region where fragments 93-XX are found is the only region that exhibits differences in fragmentation patterns between the metal bound and apo isoforms, this suggests that these residues in MntR may still have some capacity to bind metal ions under denaturing conditions. The data show that within fragment 93-111, residues 93-100 are protected from exchange upon Co$^{2+}$ binding and exchange slower than residues 102-111 (Figure 4.11).
Figure 4.11. Detailed percent deuteration exchange maps for the dimerization interface of MntR (Apo, Co, Cd, and Mn from top to bottom), residues 93-111. Fragments shown: 93-97 (apo), 93-100 (Co$^{2+}$ and Cd$^{2+}$), 93-99 (Mn$^{2+}$), and 102-111 for all isoforms. Metal-binding residues are boxed. Time-course experiments with 10 s, 30 s, 1 min, 2.5 min, 10 min, 1 h, 4 h, and 24 h sec time points (top to bottom within each experiment).
Deuterium Exchange on Cd$^{2+}$- and Mn$^{2+}$-MntR

Akin to Co$^{2+}$-MntR, the Mn$^{2+}$- and Cd$^{2+}$-MntR reconstituted forms of MntR are largely unaffected by metal binding. However, regions affected by metal-binding show a more pronounced protection from exchange compared to Co$^{2+}$-isoform (Figure 4.10). In particular, fragments 11-15 and 65-80 are significantly protected (less exchange than either apo or Co$^{2+}$-MntR), deuterating <20% within the first hour (Figure 4.8, Figure 4.9). Additionally, the Cd$^{2+}$- and Mn$^{2+}$-MntR dimer interface (93-111) also shows better protection, deuterating 20% less in the presence of Cd$^{2+}$ and Mn$^{2+}$ than either apo or Co$^{2+}$-MntR (Figure 4.11).
DISCUSSION

While crystallographic data provide crucial structural information about proteins, the dynamics of proteins in solution can often provide a complementary picture that reveals additional details of structure and function relevant under biological conditions. For example, several crystallographic studies on apo- and holo- DtxR have been reported and different crystal packing arrangements have been observed. The structures of apo- and holo- DtxR within the same space groups were found to be almost identical; however, comparing protein structures in different space groups revealed small differences between DtxR isoforms. In particular, the Cα atoms of the DNA-binding helix of one DtxR monomer shifts by ~2 Å (with respect to the dimerization core) in a hinge-like motion and twists ~2° relative to the DNA-binding helix of the other monomer. While these structural changes may contribute to the mechanism of DtxR, solution NMR studies suggest that metal binding leads to a significant disordered-to-ordered transition within the N-terminus of DtxR, enhancing the dimerization of DtxR as well as positioning the DNA-binding domains to bind DNA (12-14). Recent crystallographic evidence suggests that MntR also exhibits similar behavior, as the overall fold of various MntR isoforms appears to be nearly identical (10). We sought to examine the solution behavior of MntR in order to elucidate how metal binding contributes to the mechanism of action of MntR. Furthermore, we wanted to ascertain whether the crystal structures of MntR are indeed representative of what happens in solution, particularly in light of the temperature-dependent
discrepancies between metal sites of Mn$^{2+}$-MntR and the overall similarities between the metal-free and metal-bound structures (8, 9).

**Analysis of Apo-MntR in Solution**

By employing DXMS, we have investigated the dynamics in solution between various reconstituted forms of MntR (Figure 4.12). The exchange dynamics of apo-MntR suggest that the N-terminal domain, which contains the HTH DNA-binding motif, is more dynamic/mobile in solution than the C-terminal dimerization domain. Within MntR, residues 3-8 exhibit the most rapid exchange indicative of high mobility in solution, which is consistent with these residues being unresolved (highly disordered) in the crystallography studies (8, 9). Other regions of MntR that exchange rapidly include residues 45-52 and 54-62, which are $>$60% deuterated after one minute. The rapid exchange within these residues may be explained by examining crystal structures of MntR, which show these regions are not involved in formation of H-bonds, salt-bridges, or hydrophobic interactions. In contrast, when these stabilizing interactions are present, the initial rate of exchange is slower as observed with residues 18-27 (2 salt bridges, D27-K20 and D27-R24 Figure 4.13) and 33-42 (hydrophobic interaction of V39 with V25, V34, V42, A29, Figure 4.14).
Figure 4.12. Deuteration time course mapped onto a representation of the MntR crystal structures. Data is shown for apo, Co$^{2+}$, Cd$^{2+}$, and Mn$^{2+}$-MntR as percent deuteration and color coded in 20% increments.
Figure 4.13. Potential salt bridges between Asp27 (in green) with Lys20 and Arg24 (both in orange). Ser26, which could potentially interact with Asp27, is highlighted in magenta. Oxygen and nitrogen atoms are shown as red and blue spheres, respectively; oxygen atom from Ser26 is shown as gray sphere. A full view of MntR N-terminus (left) and a magnified view (right) are shown.

Figure 4.14. Potential interactions of Val39 (in green) with nearby hydrophobic residues (in magenta). Carbon atoms in closest contact are highlighted as yellow spheres. A full view of MntR N-terminus (left) and a magnified view (right) are shown.

While the bulk of the N-terminal domain exchanges at a similar rate, relative to the rest of the domain, fragments 11-15 and 30-32 are protected from the exchange. Notably, Tyr9 from helix 1 (located next to the protected 11-15 fragment) and Leu32 from helix 2 are buried within the N-terminal domain and are involved in hydrophobic interactions.
Formation of this hydrophobic core, with the methyl group of Leu32 positioned less than 4 Å away from and pointing directly at the phenyl ring of Tyr9 (Figure 4.15), suggests that the mobility of helix 1 and helix 2 is connected and loss of mobility in one of these helices will reduce mobility of the other. This communication between helix 1 and helix 2 may assist in the metal-mediated DNA binding, as discussed below.

Figure 4.15. Hydrophobic interactions within the N-terminal domain of MntR. Tyr9 from helix 1 is shown in red, Leu32 from helix 2 is shown in blue. Mn²⁺ ions are shown as blue spheres and are coordinated by Asp8 and Glu11 from helix 1, which are shown in magenta.

The dimer interface itself (helices 5 and 6) exhibits particularly limited exchange, even in apo-MntR, correlating well with reported biophysical studies that apo-MntR exists as a homodimer in solution (2). Within helices 5 and 6, residues 86-91 and 114-116 appear to be the most protected, with less than 40% deuteration after four hours. The
data suggest that residues within these fragments contribute to formation of a hydrophobic core that leads to dimer formation (Figure 4.16).

Figure 4.16. Dimerization interface of MntR. Phe83, Leu116, Tyr119 and Phe120 are highlighted in pink, blue, green, and red respectively. A full view of MntR (left) and a magnified view of the hydrophobic core (viewed ~90º relative to the full view, right) are shown.

Finally, helix 7, which has been suggested to be involved in initiating dimer formation of MntR, exhibits intermediate exchange rates, implying that the interactions between helix 7 of two adjacent MntR monomers might be dynamic. Nonetheless, the fact that this region does not deuterate beyond 80% is indicative of inter-helix interactions. This finding implies this region might not be the origin of dimer formation, but rather provides additional stabilization after initial dimer formation in the hydrophobic core of MntR.
Analysis of $\text{M}^{2+}\text{-MntR}$ in solution

The comparison between apo and holo MntR is summarized in Figure 4.17.

Not surprisingly, the helices that contribute metal-chelating ligands demonstrate increased protection from $^2\text{H}$ exchange, with Co$^{2+}$-bound isoform showing intermediate exchange compared to Cd$^{2+}$- and Mn$^{2+}$-MntR (Figure 4.10, Figure 4.12). The largest stabilization is observed in domain-connecting helix 4, implying that this helix is highly
mobile in solution and is significantly rigidified upon metal binding. Furthermore, the DXMS experiments suggest that within fragment 93-111, metal binding protects residues 93-100 from exchange, while residues 102-111 are more readily exchanged. In particular, residues 102-111 are exchanged more in Co\(^{2+}\)-MntR than in apo-MntR, both of which are exchanged more rapidly than either the Cd\(^{2+}\) or Mn\(^{2+}\)-MntR (Figure 4.11). This difference in deuteration appears consistent with the coordination geometries observed for these metal ions. Co\(^{2+}\)-MntR, in contrast to Cd\(^{2+}\) and Mn\(^{2+}\)-MntR, does not form a binuclear metal site within MntR. Due to lack of the second metal site (site C), His103 is not employed for metal coordination. It is possible that metal binding at site A exposes some of the residues within 102-111 region, whereas binding at site C would then offer protection for these exposed residues. Finally, helix 7 also does not exhibit changes in exchange behavior upon metal binding. This was expected as this region is far from the metal binding site.

**Mechanism of Metal Activation**

On the basis of the observation that the only regions that appear to be significantly affected (i.e. most protected from the deuterium exchange) are helix 1, 4, and 5, we propose a mechanism wherein the metal binding event serves to rigidify the MntR structure so as to reduce the mobility between the DNA-binding and dimerization domains. As previously postulated in crystallographic work (9), helix 4 that links the C-terminal dimerization domain to the N-terminal DNA-binding domain allows for mobility of the DNA-binding domains relative to the dimerization interface. These interdomain dynamics and motion of helix 4 are supported by the rapid deuteration
observed in the DXMS experiments. In good agreement with our prior hypothesis (1), the DXMS data suggest that the high mobility between the HTH domain and the dimerization interface mediated by helix 4 is restricted upon metal binding thereby rigidifying MntR to facilitate binding to DNA. However, exchange rates within the HTH motifs remain largely unaffected by metal binding, although small differences in deuteration patterns within the beta-strand region of the ‘wing’ (residues 52-62) are observed and may also contribute to the mobility of the HTH motif (Figure 4.5, Figure 4.8). This suggests that the mechanism of activation does not involve large conformational changes upon metal binding, as has been observed with Fur (6), but rather that MntR simply undergoes a structural rigidification upon binding of metal ions. Overlaying the N-terminal of Mn\textsuperscript{2+}-MntR with that of DNA-bound Co\textsuperscript{2+}-DtxR shows that the DNA-binding domains are present in very similar orientations. Because the deuterium exchange is very similar between apo and holo MntR for the DNA-binding domain, this comparison would suggest that in apo-MntR the HTH motif is already sufficiently folded to bind DNA, but that helix 4 allows for too much interdomain mobility (Figure 4.18) (9, 15). This further suggests that the role of metal binding is to merely rigidify MntR structure so as to reduce the movement of the DNA-binding domain and not alter the bulk of MntR structure.

Because the DNA-binding motif is already preformed, once the interdomain motion is constrained by the metal binding event the protein can readily bind DNA. One possible advantage of such structural locking, if the protein domains are already preorganized in solution, would be the reduced entropic cost associated with the loss of protein mobility required for the DNA-binding. In such a context, the mechanism of
DNA-binding activation of MntR presented here fits well with a simple mechanism that relies on metal binding to reduce the mobility of a preorganized protein (Figure 4.19) and is in excellent agreement with recent structural findings on apo MntR (10).

**Figure 4.18.** Overlay of residues 6-65 of MntR (PDB ID: 2F5F) and DNA-bound DtxR (PDB ID: 1F5T). MntR and DtxR are depicted as gray and cyan cartoons, DNA sequence is shown yellow ribbon. DtxR residues involved in DNA interactions are highlighted as blue sticks; corresponding residues conserved in MntR are show in red. Side-view (left) and 90° rotation (right).
Figure 4.19. Metal-mediated mechanism of MntR. Dimerization domains of MntR are shown as blue ovals and DNA-binding domains as red triangles. Activating transition metal ions capable of forming binuclear metal site are represented as green sphere while activating metal ions that form a mononuclear metal site are show as blue square. Dotted lines are used to represent motions of MntR in solution. Figure illustrates that the binuclear metal site is more efficient at rigidifying protein structure compared to a mononuclear metal site.
CONCLUSIONS

DXMS was utilized to study the solution behavior of apo and holo forms of the metalloregulatory protein MntR. This analysis has yielded new insights into the metal-mediated DNA-binding mechanism of MntR as well as evidence supporting the mechanistic conclusions drawn from recent crystallographic studies (10). Our findings suggest that metal-binding acts to conformationally restrict MntR, most notably by rigidifying helix 4, which connects the C- and N-termini of the protein. Importantly, DXMS experiments show that metal binding has only a small effect on the dynamics within the HTH DNA-binding motif. The stability of the HTH domain may be achieved by hydrophobic interactions between Tyr9 from the helix 1 and Leu 32 from helix 2 creating a hydrophobic core. Because helix 1 donates ligands for the metal binding, restricting mobility of this helix in turn causes the loss of the mobility of the hydrophobic core and hence the DNA-binding motif. In many ways the mechanism proposed for MntR is less sophisticated than other metalloregulatory proteins, but it does provide a reasonable model for the experimental findings presented here and in crystallographic studies.
MATERIALS AND METHODS

Protein Preparation

Protein was prepared as previously described (1), concentrated to \( \geq 500 \, \mu\text{M} \) and stored in 300 mM NaCl, 20 mM HEPES pH 7.2, and 5 % glycerol (storage buffer). CdCl\(_2\)·H\(_2\)O, CoCl\(_2\)·H\(_2\)O, MnCl\(_2\)·4H\(_2\)O (99.99+%) were acquired from Sigma Aldrich. Stock metal solutions were prepared and quantified as previously described (1, 2). Deuterated DXMS storage buffer was prepared identically to storage buffer, but using D\(_2\)O instead of H\(_2\)O, to give a final concentration of 90% D\(_2\)O after dilution with glycerol and pH adjustments with HCl and NaOH. Quench buffer was prepared to a final composition of 0.8% formic acid, 13.3% glycerol, and 6.2 M GuHCl. All mass spectrometry measurements were performed on a Finnigan LCQ Classic ion-trap-type mass spectrometer operated in ESI mode with spray voltage of 5000 volts, in positive ion mode, and heated capillary temperature of 200 °C. Data was acquired in centroid mode, data-dependent MS1:MS2 (collision energy 35) for peptide sequence identification, and acquired in continuous MS1 profile mode for deuterium quantification. The scan \( m/z \) range was 200-2000, allowing ready detection of peptides as small as trimers (\( m/z \approx 430 \) for the +1 charge state).

DXMS Sample Preparation

Deuterium exchange experiments were carried out by dilution of apo- or holo-MntR proteins into the deuterated DXMS buffer to initiate the on-exchange as previously described (16-25). Metal-loaded MntR was prepared by dilution of
concentrated protein stock to 120 μM MntR with either 2.15 equiv of Co²⁺ or Cd²⁺ (from 500 μM stock) or 14 equiv of Mn²⁺ (from 5 mM stock), respectively to give >90% metal bound protein (based on MntR metal binding affinities of 160 μM for Mn²⁺, 5 μM for Co²⁺, and 0.1 μM for Cd²⁺ determined in chapter 3) (26). Apo-MntR samples were diluted to 120 μM with DMXS buffer prior to initiation of exchange. All DXMS experiments were carried out at 25 ºC. ²H Exchange was initiated by dilution of 180 μL of each 120 μM MntR solution with 540 μL of deuterated DXMS buffer in microcentrifuge tubes (67.5% D₂O after addition). Exchange was monitored over the course of 24 hrs, collecting eight time points at 10, 30, 60, 150, 600, 3600, 14400, and 86400 seconds (10 s, 30 s, 1 min, 2.5 min, 10 min, 1 h, 4 h, and 24 h). At appropriate time points, 80 μL aliquots were taken out and added to 120 μL quench buffer (0.8% formic acid, 13.3% glycerol, and 6.2 M GuHCl) on ice, and flash-frozen in liquid N₂. These conditions significantly slow the rate of the exchange by lowering the pH and temperature. Deuterated samples from the time-course experiments were compared with back-exchange controls (fully-deuterated samples) to determine the extent of exchange. Back-exchange controls were prepared by dilution of MntR with 0.8% formic acid in D₂O instead of deuterated DXMS buffer and equilibrating this sample for 24 hrs at room temperature prior to chilling and quenching of exchange with quench buffer. Preliminary determination of optimal conditions of protease digestion were determined by analysis of non-deuterated samples (non-deuterated fragmentation map), where MntR was diluted into non-deuterated DXMS buffer (11, 27-33). Final sample composition prior to protease digestion was 10% protein solution, 30% deuterated
DXMS buffer, and 60% quench buffer for a total sample volume of 200 μL. Final concentration of MntR in each sample was 12 μM (40 μg protein per aliquot).

**DXMS Sample Digestion and Fragment Separation**

Flash frozen samples (containing 0.5% formic acid, 10% glycerol, and 3.7 M GuHCl) were thawed to 0 °C prior to loading onto a 66 μL column with immobilized pepsin protease at 100 μL/min for digestion as previously described (11, 27-33). Digested fragments were separated on a C18 column (Vydac, polymeric reversed phase 218 MS series) using 0.5% TFA in H₂O with a 5-45% gradient of acetonitrile over 30 min prior to injection into a Finnigan LCQ Classic mass spectrometer operating in positive ion mode. Peptide fragments generated by pepsin digestion were identified by collecting MS/MS data on non-deuterated samples, and analyzing the fragmentation data with the software package Sequest (Finnigan, Inc.). The fragments identified by Sequest were used to monitor deuterium exchange during the time-course experiments.

**DXMS Data Analysis**

Localization and quantitation of deuterium on digested fragments was performed with DXMS software from Sierra Analytics as previously described (11, 18, 27-33). An apo-MntR fragmentation map was generated to identify peptide fragments of MntR after protease digestion. Fragments generated by digestion were assigned by comparing MS data to a theoretically calculated spectrum for that fragment. Time course data was constructed by monitoring the number of deuterium atoms incorporated
into a fragment over time and converting this number to percent deuteration, as described below.

Deuterium incorporation number = (m(P) – m(N))/(m(F) – m(N))×MaxD, where m(P), m(F), and m(N) refer to the mass centroid value for partially-deuterated, fully-deuterated, and non-deuterated fragments, respectively. MaxD refers to maximum number of deuterium atoms incorporated, which is calculated by subtracting the first two residues (under assumption that these amino acids do not retain deuterium) and the number of prolines from the total number of residues in a fragment (11, 18). Because the first two amino acids on each digested fragment can not be conclusively assigned, they are omitted from the deuteration analysis. Percent deuteration of MntR was calculated as the ratio of 100×(deuterium incorporation number)/MaxD (11, 18). Percent deuteration values were interpreted by mapping either onto the MntR sequence (“ribbon maps”) or representations of the MntR crystal structures to analyze exchange dynamics on the protein.

The text of chapter 4, in full, is a reprint of the material published in the Journal of Biological Inorganic Chemistry, 2007 “Conformational studies of the manganese transport regulator (MntR) from Bacillus subtilis using deuterium exchange mass spectrometry”. The dissertation author was the primary researcher and/or author and the co-authors (Li S, Woods V Jr, Cohen SM) listed in this publication contributed to or supervised the research which forms the basis for this chapter. The authors thank Prof. Arthur Glasfeld (Reed College) for sharing crystallographic data and helpful
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Chapter 5.

Discussion
DISCUSSION

We have demonstrated the ability of MntR to respond differently to a toxic metal ion (Cd$^{2+}$) compared to an essential metal ion (Mn$^{2+}$) and described the potential significance of this observation under physiological conditions. In conjunction with crystallographic evidence from other research groups, we have provided strong evidence that formation of the binuclear metal site is necessary for full activation of the DNA-binding ability of MntR. The binuclear metal site appears more efficient than a mononuclear site at rigidifying the structure of MntR, likely lowering the potential entropic cost associated with DNA-binding.

As no bacterial Mn$^{2+}$ metallochaperones have been identified, it is astonishing that MntR is able to respond specifically to this metal ion. Comparison of the metal binding affinities of MntR clearly demonstrates that MntR binds Mn$^{2+}$ with the weakest thermodynamic affinity out of the various metal ions tested in this thesis. Thus, it is not the metal-binding affinities that allow MntR to be Mn$^{2+}$-specific. We have suggested some determinants of metal specificity; in particular, we propose that in the cell, Mn$^{2+}$ is one of the few transition metal ions accumulated to concentrations at which MntR would be able to bind this ion. In contrast, transition metal ions for which MntR exhibits high affinities are not intracellularly available in sufficient concentrations to result in metal ion binding. Alternative explanations for Mn$^{2+}$ specificity might lie in the kinetics of formation of binuclear Mn$^{2+}$ metal site compared to mononuclear metal site observed with non-native transition metal ions. In addition, utilization of an unidentified Mn$^{2+}$-metallochaperone to deliver this ion directly into MntR might also
explain the Mn$^{2+}$-specificity of MntR. More studies are necessary to determine how these factors come together to facilitate trafficking of manganese in biology.

Our investigations are consistent with an emerging theme in metalloregulatory proteins wherein the metal activation profile does not necessarily correlate with the metal-binding affinities, but rather is dependent on metal site geometry and stoichiometry (1). While this trend explains why the binuclear metal site is responsible for allowing MntR to selectively repress DNA in presence of Mn$^{2+}$, it certainly is curious whether MntR can be erroneously loaded with a tighter-binding non-native metal ion and what the ramifications of this event might be. Our experiments with the single site mutants MntR E99C and MntR D8M demonstrate that while the metal affinities remain largely unperturbed, the DNA-binding affinities can vary significantly. While we suspect that this behavior stems from altered coordination in the metal-binding site, the determinants that guide this selectivity are worth investigating as they may prove useful in both protein engineering and ligand design in inorganic chemistry.
FUTURE DIRECTIONS

While we have investigated numerous aspects of MntR behavior, most of our experiments focused on the determination of thermodynamic parameters (e.g. metal and DNA affinities, protein stabilization upon metal binding). It would be interesting to determine the kinetics of MntR metal binding and see whether the kinetic parameters affect the proposed mechanism of action of MntR. As has already been demonstrated, strong metal binding affinities do not always result in activation of DNA-binding – one potential explanation being that it is the coordination geometry imposed by the metal ion that dictates its ability to activate for DNA binding. Determining which type of MntR metal site (mononuclear vs binuclear) is more inert could also shed light on why MntR is only responsive to the native metal ions in vivo. In particular, if the binuclear site of MntR is more inert compared to the mononuclear site, the strong metal binding of metal ions that form the mononuclear site might not persist long enough to result in structural rigidification needed for DNA-binding.

In addition to probing the kinetics of the metal site formation, other interesting aspects of the binuclear site of MntR should be investigated. From a bioinorganic perspective, mutations of metal-chelating amino acids with alanine residues to create and investigate mononuclear site A and site C independently may shed light as to what is the “minimal” metal site needed to initiate DNA binding. For example, is formation of a mononuclear site C in itself sufficient for DNA-binding? Furthermore, it would interesting to test whether MntR proteins with a mononuclear metal site can be further
loaded with an additional metal ion, so as to recover the binuclear metal site and to test whether the ability bind DNA is fully recovered under such conditions.

Another interesting aspect of MntR behavior is the DNA-specificity of this protein. Comparison of the DNA-binding affinities for the *mntH* and *mntA* oligonucleotides suggests that affinities are similar for these sequences, and in some cases the *mntA* sequence is preferred. The fact that MntR is able to respond differently to these two sequences might be inherent in the oligonucleotide sequence, or due to the positioning of the DNA-binding motifs of MntR with respect to the oligonucleotide sequences, or both. It would be interesting to compare the DNA affinities of MntR for various DNA sequences to determine the optimal sequence that MntR could bind. In particular, focusing on native sequences vs recognition sequences of other non-Fe^{2+} responsive members of the DtxR family might show how this protein evolved to possess its DNA-specificity.

Finally, a number of potentially Mn^{2+}-responsive metalloregulatory proteins have not been fully characterized. In particular, biochemical and biophysical investigations with TroR, SeaR, and SirR proteins would help not only to gain better understanding different mechanisms in bacterial Mn^{2+} homeostasis, but also the bioinorganic origins of metal selectivity for this metal ion (2-4).
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