Bioinorganic Chemistry of the Human Host-Defense Protein
Calprotectin

by

Megan Brunjes Brophy

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Signature of Author:___

Signature redacted

Department of Chemistry
May 8, 2015

Signature redacted

Certified by:_______

Elizabeth M. Nolan
Associate Professor
Thesis Supervisor

Signature redacted

Accepted by:_______

Robert W. Field
Haslam and Dewey Professor of Chemistry
Chairman, Departmental Committee for Graduate Studies
This doctoral thesis has been examined by a committee of the Department of Chemistry as follows:

Signature redacted

Catherine L. Drennan
HHMI Professor and Investigator and Professor of Chemistry and Biology
Committee Chairperson

Signature redacted

Elizabeth M. Nolan
Associate Professor of Chemistry
Thesis Supervisor

Signature redacted

JoAnne Stubbe
Novartis Professor of Chemistry and Professor of Biology
Committee Member
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Abstract

The human innate immune system responds to bacterial and fungal pathogens by
releasing the metal-chelating protein calprotectin (CP) at sites of infection and in the upper
layers of the epidermis. CP is a Mn(II)- and Zn(II)-binding protein. The work described in this
thesis elucidates the metal-binding properties of CP, and correlates these properties with in
vitro growth inhibition of bacteria and fungi. We report that the metal-binding properties of
CP are modulated by Ca(II), and we propose a working model in which CP responds to
physiological Ca(II)-ion gradients to become a potent Zn(II)- and Mn(II)-chelating agent in
the extracellular space. Individual chapter summaries follow.

Chapter 1: Bioinorganic Chemistry of the Host Pathogen Interaction

Transition metal ions are required for all forms of life. During the course of infection,
pathogenic microorganisms must acquire transition metals from the host. Three metals of
interest from this standpoint are iron, zinc, and manganese. This chapter describes bacterial
metal-ion homeostasis machineries, and metal-requiring processes with a focus on Zn(II)
and Mn(II). This chapter then highlights the S100 family of Ca(II)-binding proteins and
discusses the Zn(II)-, Cu(II)-, and Mn(II)-binding properties of S100B, S100A12, S100A7,
S100A15, and S100A8/S100A9. Finally, an overview of the scope of this thesis is
presented.
Chapter 2: Calcium Ion Gradients Modulate the Zinc(II) Affinity and Antibacterial Activity of Human Calprotectin

Calprotectin (CP) is a human neutrophil protein that is produced and released by neutrophils at sites of infection, where it prevents the growth of microorganisms by sequestering bioavailable zinc(II) and manganese(II). In this chapter, we present metal-binding studies to elucidate the Zn(II)-binding properties of CP. We report unique optical absorption and EPR spectroscopic signatures for the interfacial His$_3$Asp and His$_4$ sites of human CP by using Co(II) as a spectroscopic probe. Zinc competition titrations employing colorimetric and fluorimetric Zn(II) sensors establish that CP coordinates two Zn(II) ions / CP heterodimer. The Ca(II)-insensitive Zn(II) sensor ZP4 is used to determine the $K_d$ of CP for Zn(II) in Ca(II)-deplete and Ca(II)-replete conditions. These competition titrations afford apparent $K_{d\text{,site1}} = 133 \pm 58$ pM and $K_{d\text{,site2}} = 185 \pm 219$ nM in the absence of Ca(II). In the presence of excess Ca(II) these values decrease to $K_{d\text{,site1}} \leq 10$ pM and $K_{d\text{,site2}} \leq 240$ pM. In vitro antibacterial assays indicate that the metal-binding sites and Ca(II)-replete conditions are required to inhibit the growth of Gram-negative and Gram-positive bacteria. We propose a model in which Ca(II) ion gradients modulate the antibacterial activity and Zn(II)-binding properties of human CP.

Chapter 3: High-Affinity Manganese Coordination by Human Calprotectin Is Calcium-Dependent and Requires the Histidine-Rich Site at the Dimer Interface

In this chapter, we report that the His$_4$ motif at the S100A8/S100A9 dimer interface of CP is required for high-affinity Mn(II) coordination. We identify a low-temperature EPR spectroscopic signal for this site that is consistent with high-spin Mn(II) in an octahedral coordination sphere. This site could be simulated with zero-field splitting parameters $D = 270$ MHz and $E/D = 0.30$ ($E = 81$ MHz). This analysis, combined with studies of mutant
proteins, suggests that (A8)His17, (A8)His27, (A9)His91, (A9)His95 and two as-yet unidentified ligands coordinate Mn(II) at site 2. These studies support a model in which CP responds to Ca(II) ion gradients to become a potent metal-ion chelator in the extracellular space.

Chapter 4: Contributions of the C-terminal Tail of S100A9 to High-Affinity Manganese Binding by Human Calprotectin

This chapter examines the role of the S100A9 C-terminal tail to high-affinity Mn(II) coordination by human CP. We present a 16-member mutant family with mutations in the S100A9 C-terminal tail (residues 96-114), which houses three histidine and four acidic residues, to evaluate its contribution to Mn(II) sequestration. These studies confirm that two His residues at positions 103 and 105 complete the octahedral coordination sphere of CP in solution.

Appendix 1: Sequence Alignments of Transition-Metal Binding S100 Proteins

Sequence alignments of S100A7, S100A8, S100A9, S100A12, S100A15, and S100B proteins from multiple organisms are presented.

Appendix 2: Characterization of CP Mutant Proteins by Circular Dichroism and Analytical Size Exclusion Chromatography

Additional characterization of CP and mutant proteins employed in Chapters 2-4 is presented.

Appendix 3: Structures of Sensors Used In this Work

The structures of Zincon, MagFura-2, Zinpyr-1, and Zinpyr-4 are presented.
Appendix 4: Manganese Binding Properties of Human Calprotectin under Conditions of High and Low Calcium

This appendix represents a collaborative work with the Drennan Lab (MIT) and Britt Lab (UC Davis) to study the Mn(II)-CP complex in low- and high-Ca(II) conditions. We report a crystal structure of Mn(II)-, Ca(II)-, and Na(I)-bound CP with Mn(II) exclusively coordinated to the His$_6$ motif. Electron spin-echo envelope modulation and electron-nuclear double resonance experiments demonstrate that the six coordinating histidine residues are spectroscopically equivalent. The observed $^{15}$N ($I = \frac{1}{2}$) hyperfine couplings ($A$) arise from two distinct classes of nitrogen atoms: the coordinating $\epsilon$-nitrogen of the imidazole ring of each histidine ($A = [3.45, 3.71, 5.91]$ MHz) and the distal $\delta$-nitrogen ($A = [0.11, 0.18, 0.42]$ MHz). In the absence of Ca(II), the affinity of CP for Mn(II) drops by two to three orders of magnitude, and Mn(II) coordinates to the His$_6$ site as well as other sites on the protein.
For Maggie
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The first person I need to acknowledge is my graduate advisor, Elizabeth Nolan. I first met Liz during my MIT visit weekend in spring 2010. For the Friday night dinner all of the biological prospective students, as well as several faculty members, walked from the Marriott hotel in Kendall Square to Simmons. Somehow, probably because she was walking the fastest, Liz ended up in front. I wanted to talk to her about her research program and interests, so I propelled myself to the front of the group. I don’t actually remember what I asked her, but I do remember that Liz was walking so quickly I had to jog every few strides to keep up. After I came to MIT and Liz became my research adviser, we worked together to get the calprotectin project off the ground. Things move quickly in a new lab, and there were several times during the first few years that I still felt like I was running to keep up with the pace. Fortunately, Liz doesn’t leave people behind. Thank you, Liz, for being a mentor to me over the last five years. I have grown and changed so much as a scientist and as a person, and I am truly grateful for the opportunity to work with you.

The work presented in this thesis details studies with at least 34 proteins (including mutants), many of which have been prepared multiple times over the years. This work represents the efforts of many people, including Dr. Joshua Hayden, Lisa Cunden, Aleth Gaillard, Toshiki Nakashige, and Hope Flaxman. Joshua and I worked together on the initial phases of the calprotectin project, and he performed the Zn(II) competition assays, low-temperature Mn(II) EPR, and determined the affinities of CP for Zn(II) and Mn(II). Joshua also taught me almost everything I know about EPR, and he provided valuable assistance in interpreting the low-temperature Co(II) EPR data. Lisa Cunden purified and characterized the CP-Ser(H27D) mutant. Aleth, Toshiki, and I worked on the C-terminal tail aspect of the project. They both purified and characterized many proteins. Aleth also performed Ca(II)
titrations with CP-Ser-AAA and CP-Ser-\(\Delta 101\) mutants, and Toshiki assisted with the collection of EPR data.

One of the best things about graduate school has been that daily I come into lab and I get to work with some of the smartest, kindest, and most talented people that I have ever met. Tengfei, Haritha, Piotr, Yoshi, Joshua, Andy, Lisa, Aleth, Yunfei, Jill, Toshiki, Simone, Fabien, Julie, Sumin, Hope, I-Ling, Shion, Phoom, Jules, Rose, Anmol, Fangting, Tim, Justin and Claire (in no particular order) helped make even the rough days fun and enjoyable. Joshua was the first post-doc to work on the calprotectin project with me, and we worked closely on the first two projects. Joshua’s good nature and sense of humor made all of those long days collecting EPR data, trouble shooting the EPR, trying to get the cryostat to cool down (and stay cool!!) actually fun and full of much-needed laughter. Lisa was the first undergraduate who worked with me on my ‘baby’ protein, and I’m so glad that I was able to con her into coming back to keep working on S100 proteins. Justin always had a smile on his face, and was always ready to listen Disney music and Taylor Swift. Andy, Jill, and Tengfei became my friends and confidants, and the week they all moved on to the next stage of their careers was one of the saddest. The best thing about science is the people that you get to work with, and the worst is eventually saying goodbye to them. Phoom. Phoom. Phooooom. No, Phoom. Haritha, it has been so wonderful to get to know you over the years. Thanks for the Indian food, and for trying to make it “slightly” less spicy for my poor English tastebuds. I’m glad that my attempts to eat spicy food gave you something to laugh about. Fabien, Simone, and Julie have been great hiking buddies for trips up to the White Mountains. Jules knows an Archer quote for every situation and we shared many conversations during the Serial craze of 2014. Hope is an undergraduate who has worked with me on S100A7 and S100A15, and it has been a joy to have her in lab.

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I also want to take this opportunity to acknowledge my collaborators over the years: Derek Gagnon, Troy Stich, and Dave Britt performed advanced EPR spectroscopic measurement on Mn(II)-CP, and Sarah Bowman and Cathy Drennan have worked on the crystal structure of Mn(II)-, Ca(II)- and Na(I)-bound CP. Cathy, in addition to being a wonderful collaborator, is also my thesis chairperson and has given me valuable advice over the years. Mickie Killian and Thomas Brunold have performed MCD experiments on Co(II)-bound CPs and helped to provide valuable insight into the coordination sphere at the His$_6$ site.

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“A man would make but a very sorry chemist if he attended to that department of human knowledge alone.” Mary Shelley, Frankenstein, 1818

“Be of good cheer. If science teaches us anything, it teaches us to accept our failures, as well as our successes, with quiet dignity and grace.” Mel Brooks and Gene Wilder, Young Frankenstein, 1974
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Chapter 1: Bioinorganic Chemistry of the Host Pathogen Interaction

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1.1 Introduction

During the course of infection, pathogenic microorganisms must acquire essential nutrients, including transition metal ions, from the host.\textsuperscript{1-4} This chapter summarizes metal-ion uptake systems that are employed by microorganisms, and the utilization of these metals with a focus on zinc(II) and manganese(II). This chapter will also describe the mammalian systems that are employed to sequester these essential nutrients from pathogenic bacteria and fungi.

1.2 Iron and Nutritional Immunity

Nearly all microorganisms require iron for growth, and iron is employed in a variety of enzyme cofactors including hemes, non-heme iron centers, and iron-sulfur clusters. Microbes may acquire iron by employing membrane-embedded transporters and by using small-molecule chelators to scavenge iron from the host.\textsuperscript{5-9} Moreover, \textit{Neisseria meningitidis} is able to pirate Fe(III) from the human iron-binding protein transferrin,\textsuperscript{10} and \textit{Staphylococcus aureus} and \textit{Mycobacterium tuberculosis} import heme from the host as an iron source.\textsuperscript{11,12} The human host proteins transferrin, lactoferrin, and siderocalin (also called lipocalin-2 or neutrophil gelatinase-associated lipocalin (NGAL)) deplete bioavailable iron in blood serum, secretory fluids, and sites of infection to limit the pool of iron available to microorganisms (Figure 1.2.1).\textsuperscript{13-15} Transferrin and lactoferrin coordinate Fe(III) with high affinity ($K_d \approx 10^{-20}$ M),\textsuperscript{16,17} and siderocalin captures and hydrolyzes ferric-bound enterobactin.\textsuperscript{18}

Iron provides a historical paradigm for the importance of metals at the host pathogen interface. The sequestration of iron by the innate immune system and iron homeostasis mechanisms, both microbial and mammalian, are relatively well understood and provide a basis for subsequent studies with manganese and zinc.
Figure 1.2.1. Structures of siderocalin and lactoferrin. (A) and (B) show human siderocalin with hydrolyzed ferric enterobactin. (PDB ID: 1L6M). (C) and (D) show diferric human lactoferrin, and an expansion of Fe(III) coordinated to the C-terminal lobe is shown in D. (PDB ID: 1LFG).
1.3 Acquisition and Utilization of Zn(II) by Microorganisms

1.3.1 Zn(II) Uptake by Bacteria

Bacteria employ a large arsenal of proteins to maintain an appropriate level of cytoplasmic zinc, and zinc acquisition is a virulence factor of many bacteria including *Salmonella enterica* serovar Typhimurium,\(^{20-22}\) *Escherichia coli*,\(^{23-26}\) *Campylobacter jejuni*,\(^{27,28}\) *Pseudomonas aeruginosa*,\(^{29}\) *Yersinia pestis*,\(^{30}\) *Acinetobacter baumanii*,\(^{31,32}\) *Moraxella catarrhalis*,\(^{33}\) *Vibrio parahaemolyticus*,\(^{34}\) *Listeria monocytogenes*,\(^{35}\) *Streptococcus pneumoniae*,\(^{36}\) *Brucella abortus*,\(^{37,38}\) *Haemophilus influenzae*,\(^{39}\) *Proteus mirabilis*,\(^{40}\) and *Haemophilus decreyi*.\(^{41}\) A summary of the Zn(II) homeostasis machinery that has been identified in *E. coli* is shown in Figure 1.3.1. In general, the Zn(II)-uptake systems are regulated at the transcriptional level by the zinc-uptake regulator Zur, and the Zn(II) exporters are regulated by the zinc transport regulator ZntR (*vide infra*).

The primary high-affinity Zn(II) importer of many bacteria is the three-component ATP-binding cassette ZnuABC.\(^{21,22,26}\) This transporter is typically embedded in the inner membrane of Gram-negative bacteria and consists of a periplasmic Zn(II)-binding lipoprotein (ZnuA), a dimeric transmembrane permease (ZnuB), and a cytoplasmic ATP-hydrolase (ZnuC). ZnuABC facilitates the active import of Zn(II) by coupling ion transport to ATP hydrolysis; however, the molecular details of the metal-ion transfer are not well understood and there is no available structure of the intact transporter. Moreover, Gram-negative bacteria such as *E. coli* have an outer membrane that acts a permeability barrier. To the best of our knowledge, no outer-membrane Zn(II) transporters have been identified for *E. coli*, and it is generally believe that Zn(II) diffuses across the outer membrane.
Figure 1.3.1. Zn(II)-homeostasis machinery in *E. coli*. Zinc(II) diffuses across the outer membrane into the periplasm. Periplasmic Zn(II) is transported across the inner membrane into the cytoplasm by ZnuABC, ZinT, and ZupT. The Zn(II)-binding transcriptional regulator proteins Zur and ZntR coordinate Zn(II) and regulate the transcription of *znuA*, *znuCznuB*, and *zinT* or *zntA*, respectively.

The periplasmic solute-binding protein ZnuA is soluble, and there are several available structures of ZnuA from different organisms including *E. coli*, *Salmonella*, and *Synechocystis* 6803. ZnuA is a Cluster 9 solute-binding protein that is characterized by two \((\alpha/\beta)_4\) lobes that are linked by a backbone helix. The *E. coli* and *Salmonella* ZnuA proteins contain a conserved disulfide bond in the C-terminal lobe domain. The role of this disulfide is unknown, and it may contribute to the stability of ZnuA or regulation of Zn(II) binding. The two structures of *E. coli* ZnuA reveal a primary Zn(II)-binding site in the cleft between the two lobes that coordinates Zn(II) in a tetrahedral geometry at a His\(_3\)Glu coordination motif.
addition to the primary Zn(II)-binding site, ZnuA has a His-rich loop that is disordered in the available crystal structures (Figure 1.3.2); however, this region likely contributes to Zn(II) binding by ZnuA. Indeed, *E. coli* ZnuA outcompeted MagFura-2 ($K_{d,Zn(II)} = 20$ nM) for 2 equivalents of Zn(II) / ZnuA, confirming the presence of a second high-affinity Zn(II) site. The flexible nature of the His-rich loop has made it difficult to resolve crystallographically, and its role in Zn(II) binding is not known. This loop may facilitate Zn(II) transfer to the ZnuB subunit, sense high levels of periplasmic Zn(II), or assist in recruitment of Zn(II) from the periplasm.42,46,47

Gram-negative organisms including *E. coli* and *Salmonella* express an additional lipoprotein called ZinT that is involved in Zn(II) uptake.48-53 ZinT may contain one or two Zn(II)-binding sites depending on the bacterium. The *Salmonella* ZinT is regulated by Zur and contributes to growth in Zn(II)-deplete media; however, mutation of *zinT* did not attenuate the virulence of *Salmonella* in mice.48 Regardless, ZnuA and ZinT form a 1:1 complex in the presence of Zn(II). Later studies determined that ZinT coordinates Zn(II) with $K_d = 22 \pm 2$ nM, and that the Zn(II)-bound form of ZinT is sufficient for complex formation with ZnuA.50 On the basis of these studies, it has been proposed that ZinT acts as a Zn(II) chaperone for ZnuA in the periplasm.48 Gram-negative bacteria also employ the ZIP-family member ZuP'T for growth in Zn(II)-deficient conditions.23,54,55
Figure 1.3.2. Sequence alignment of Zn(II)-coordinating lipoproteins from E. coli, S. pneumoniae, and S. aureus. Zn(II)-coordinating residues are shown in bold. The ZnuA-like proteins/domains are highlighted in orange, and the ZinT-like proteins/domains are highlighted in blue. A histidine-rich loop that is characteristic of Zn(II)-solute binding proteins is indicated by a bold line.
The Zn(II)-uptake machinery of Gram-positive bacteria differs from that of Gram-negative bacteria and is, in general, not well understood. *S. pneumoniae* is one human pathogen that has been the focus of recent work on Zn(II) transport. S. pneumoniae acquires Zn(II) through an ABC transporter called AdcABC. By analogy to *E. coli* ZnuABC, AdcA is an extracellular solute-binding protein, AdcB is a transmembrane permease, and AdcC is an ATP hydrolase. The amino acid sequence of AdcA reveals two Zn(II)-binding domains: an N-terminal ZnuA-like domain, and a C-terminal ZinT-like domain (Figure 1.3.2). *S. pneumoniae* also expresses a second Zn(II)-binding lipoprotein, AdcAll, that is homologous to ZnuA and lacks the ZinT-like domain. Both AdcA and AdcAll are employed for Zn(II) uptake via AdcBC. Additional polyhistidine triad (PhT) proteins appear to deliver Zn(II) to AdcAll.

Zinc(II) uptake by other Gram-positive pathogens is not as well understood. For example, *S. aureus* appears to utilize a zinc uptake regulator protein; however, it is not known how *S. aureus* acquires Zn(II) from the environment. Putative AdcB/AdcC homologues have been identified as mreA and mreB, but they do not appear to be transcribed in Zn(II)-deficient conditions. A BLAST search revealed that the *S. aureus* genome encodes an AdcA homolog (Figure 1.3.2), but this gene does not appear to be part of a zinc-transport operon, and it is not clear how its transcription is regulated. In a mouse model of *S. aureus* infection, Zn(II) is depleted from abscesses, however, it is currently unclear how *S. aureus* may compete with the host for Zn(II). *S. aureus* has been employed as a model organism to study metal-ion sequestration by the host, and it will be important to elucidate staphylococcal Zn(II) homeostasis mechanisms and requirements.

Bacteria concentrate Zn(II) from *in vitro* growth media, however, excess cytoplasmic Zn(II) is toxic to bacterial cells. Zn(II) efflux proteins include *E. coli* ZntA, a P-type ATPase that actively transports Zn(II), and ZitB, a cation diffusion facilitator protein (Figure 1.3.1). ZntA confers resistance to the toxic effects of the metals Zn(II), Pb(II), and
Cd(II). Each ZntA protein binds two equivalents of Zn(II) with $K_a = 10^8 \text{ M}^{-1}$. Recent structural studies of apo ZntA from *Shigella sonnei* supports the presence of a high-affinity site in the transmembrane domain, and a second high-affinity site is likely located in the N-terminal cytoplasmic domain. The cation diffusion facilitator proteins ZitB and Yiip are H$^+$ / Zn$^{2+}$ antiporters that contribute to Zn(II) resistance of *E. coli*. Zn(II) homeostasis is tightly regulated by *E. coli* cells. The transcription of Zn(II)-uptake proteins, including ZnuABC and ZntT, is regulated by the metalloregulatory protein Zur (Figures 1.3.1 and 1.3.3). A crystal structure of *E. coli* Zur bound to its cognate DNA sequence was recently reported, and reveals that two Zur dimers bind to this DNA fragment. Zur has a femtomolar affinity for Zn(II), and the Zn(II)-bound form of Zur binds to DNA and represses the transcription of Zn(II)-uptake proteins. Zn(II) efflux is upregulated by the Zn(II)-bound form of ZntR, which also chelates Zn(II) with femtomolar affinity. Recent studies indicate that ZntR responds to nanomolar concentrations of chelatable Zn(II). The high affinity of these proteins for Zn(II) supports the notion that the concentrations of "free" Zn(II) within the bacterial cell are tightly regulated.

Figure 1.3.3. Crystal structure of Zur bound to cognate DNA (PDB ID: 4MTD). The two dimers are shown in purple/pink and green/sea foam. The duplex DNA is colored grey, and Zn(II) ions are shown as chocolate spheres.
1.3.2 Additional Zn(II) Transport Systems

The Zn(II)-homeostasis proteins above are found in a variety of bacteria and are relatively well studied. *E. coli* and *S. Typhimurium* also express a ZIP-family member named ZupT that contributes to Zn(II) acquisition and virulence.\(^{23,34,55}\) *N. meningitidis* expresses a TonB-dependent membrane protein, ZnuD, in Zn(II)-deficient conditions that may be utilized for zinc or heme import.\(^{73-75}\) *N. meningitidis* may also acquire Zn(II) by pirating zinc from the human zinc-binding protein calprotectin.\(^{76}\) The fungal pathogen *Aspergillus fumigatus* requires the Zn(II) transporter ZrfC for virulence,\(^{77}\) and *Candida albicans* scavenges Zn(II) with the extracellular protein Pra1.\(^{78}\) The *Y. pestis* siderophore yersiniabactin also contributes to Zn(II) acquisition.\(^{30}\) The Zn(II)-homeostasis mechanisms of many pathogens remain unexplored, and it is likely that additional Zn(II)-uptake and -efflux systems will be discovered in the future.

1.3.3 Microbial Utilization of Zn(II)

Zinc is essential cofactor for many proteins and may be important for either structural integrity or reactivity. The *E. coli* 70S ribosome coordinates up to 8 equivalents of Zn(II).\(^{79,80}\) It is unclear why these ribosomes concentrate Zn(II); however, it has been proposed that these ribosomes may be used as a rainy day Zn(II) store.\(^{81}\) There are several bacterial virulence factors that require Zn(II) as a cofactor. Metallo-β-lactamases require Zn(II) as a cofactor,\(^{82-85}\) and metalloproteinases such as *S. aureus* aureolysin require Zn(II).\(^{86,87}\) Moreover, the Zn(II)-enzyme LasB is required for *Pseudomonas* swarming,\(^{88}\) and Zn(II) modulates envelope stress in enteropathogenic *E. coli*.\(^{89}\) Zn(II) may also influence the formation of *S. aureus* and *E. coli* biofilms;\(^{52,90}\) however, the molecular basis for this phenomenon is not well understood. In summary, bacteria utilize Zn(II) as an essential cofactor in many enzymes; however, it is not always clear how Zn(II) contributes to pathogen survival and virulence in the mammalian host. It is likely that Zn(II) plays a
multifaceted role in bacterial pathogenesis, and additional work will need to be done to clarify the molecular role of Zn(II) in many organisms.

1.4. Manganese Uptake and Utilization by Bacteria

1.4.1 Manganese Uptake by Bacteria

Animal models of infection suggest that Mn(II) uptake systems are required for virulence in a variety of bacterial pathogens including *S. aureus*, *S. pneumoniae*, *Borrelia burgdorferi*, *S. Typhimurium*, and *Y. pestis*. The mechanisms of Mn(II) acquisition by microbes as well as the microbial processes and virulence factors that require Mn(II) are an active area of current research initiatives (Figure 1.4.1).

![Figure 1.4.1 Model of Mn(II) homeostasis in S. aureus and examples of some Mn(II)-containing enzymes.](image-url)
Bacteria primarily rely on two classes of Mn(II)-importers, Nramp-type transporters and ATP-binding cassette (ABC) importers, to shuttle divalent manganese into the cytoplasm.\textsuperscript{96} The Nramp-type transporters (e.g. MntH from \textit{S. aureus}, Figure 1.4.1) are comprised of multiple membrane-embedded helices. A paucity of structural or biochemical information about these transporters is available,\textsuperscript{97} making studies of Nramp-type machinery a rich area for exploration. One recent study reports the crystal structure of ScaDMT from \textit{Staphylococcus capitis}, and represents the first structural information for this family of transporters.\textsuperscript{98} The ABC-type importers are three- or four-component systems comprised of (i) a soluble extracellular or periplasmic binding protein, (ii) a transmembrane permease, and (iii) a cytoplasmic ATP hydrolase. Noteworthy examples of ABC-type importers that are expressed by pathogens for Mn(II) acquisition include PsaABC of \textit{S. pneumoniae} and MntABC of \textit{S. aureus}. In recent years, structural and biochemical studies of the solute-binding proteins from \textit{S. pneumoniae} (PsaA) and \textit{S. aureus} (MntC) have informed the mechanism of bacterial Mn(II) capture.\textsuperscript{99-102} Structural studies revealed that various Mn(II) solute-binding proteins display nearly identical secondary and tertiary structures.\textsuperscript{99,100,103-105} These solute-binding proteins are characterized by two \((\alpha/\beta)_4\) lobes that are linked by a backbone \(\alpha\)-helix (Figure 1.4.2). In order to concentrate metals from the environment into the cytoplasm, solute-binding proteins must coordinate a cognate metal with high affinity and subsequently deliver the metal to the transmembrane protein. Because no Mn(II) solute-binding protein has been crystallized with its transmembrane partner, the mechanism of metal ion release into the transmembrane protein remains unclear.
Figure 1.4.2 Structures MntC (A) and (B) and PsaA (C)-(E). The Mn(II)-bound monomer of S. aureus MntC and an expansion of the Mn(II)-binding site are shown in (A) and (B). PDB: 4K3V.99 (C) Overlay of apo PsaA (light blue), Mn(II)-bound PsaA (lavender), and Zn(II)-bound PsaA (green). (D) and (E) Overlays of the Mn(II)-binding site of PsaA with apo PsaA (D) and the Zn(II)-binding site of Zn(II)-bound PsaA (green). Apo PsaA, PDB: 3ZK7;102 Mn(II)-bound PsaA, PDB: 3ZTT;100 Zn(II)-bound PsaA, PDB: 1PSZ.106

S. pneumoniae PsaA is a pneumococcal virulence factor, and small-molecule Mn(II)-uptake inhibitors are a promising target for drug design.107 Pneumococcal PsaA (34.6 kDa) has been crystallized in the apo, Mn(II)-bound, and Zn(II)-bound forms (Figure 1.4.2 panels C-E).100,102,106 The structure of Zn(II)-PsaA is of interest because Zn(II) is toxic to S. pneumoniae at high concentrations. Zn(II) inhibits Mn(II) uptake by the PsaABC system, which is attributed to Zn(II) coordination at the Mn(II) site of PsaA.58,108 PsaA also coordinates Cd(II), and Cd(II) competes with Mn(II) for PsaA.109 The overall fold of PsaA is very similar in the apo, Zn(II)- and Mn(II)-bound structures, but the length of the backbone helix varies between the apo and metal-bound forms. In apo PsaA, the backbone helix extends an additional turn, indicating that Zn(II) and Mn(II) coordination induces unwinding of this helix as the lobe domains change conformation.102 Zn(II) and Mn(II) are both
coordinated by His67, His139, Glu205, and Asp280 (Table 1.4.1, Figure 1.4.2 panels D and E).\textsuperscript{100,106} Zn(II) and Mn(II) coordination result in similar changes to the secondary structure of PsaA and the protein adopts a “closed” conformation with both metals. PsaA coordinates Zn(II) in a tetrahedral geometry with Glu205 and Asp280 each coordinating Zn(II) in a monodentate manner (Table 1.4.1). An overlay of Mn(II)- and Zn(II)-bound PsaA suggests that there is a small rearrangement of the metal-binding residues between the two forms. The Mn(II) coordination sphere of Mn(II)-PsaA has been described as four-coordinate;\textsuperscript{100} however, Glu205 and/or Asp280 may afford bidentate interactions (Table 1.4.1). Additional biophysical studies are needed to address this ambiguity and to fully elucidate the molecular basis for how PsaA facilitates Mn(II) delivery to its transmembrane partner PsaC.

The staphylococcal solute-binding protein MntC (35 kDa) is expressed during infection, and is a current target for the development of a multivalent vaccine against S. aureus.\textsuperscript{91,110} MntC was recently crystallized with a putative Mn(II) ion in the metal-binding pocket (Figure 1.4.2 panels A and B).\textsuperscript{99} The Mn(II)-binding site is similar to that of PsaA and formed by His50, His123, Glu189, and Asp264 (Figure 1.4.2, Table 1.4.1); however, the Mn(II) center is five-coordinate. Asp264 provides a bidentate ligand whereas a monodentate interaction is observed for Glu189. Isothermal titration calorimetry (ITC) experiments performed in the presence of the Mn(II) competitor citrate ($K_d,\text{Mn(II)} \approx 10^{-4}$ M) revealed that MntC binds Mn(II) with nanomolar affinity ($K_d = 4.4 \pm 0.9$ nM at 25 °C and pH 6.0)\textsuperscript{99} MntC also coordinates Zn(II) and Cd(II). In contrast to Mn(II), binding of these divalent metal ions appears to be irreversible and may render the transporter inactive.\textsuperscript{99} The irreversibility of Zn(II) binding is similar to the behavior reported for PsaA,\textsuperscript{102} and indicates that elevated levels of Zn(II) may also shut down the MntABC transporter machinery.
Table 1.4.1. Metal-ligand bond distances in selected Mn(II) solute-binding proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Residue / Coordinating atom</th>
<th>Metal-Ligand Bond Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn(II)-MntC&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>His50 / Nε2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>His123 / Nε2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Glu189 / Oε1</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Glu189 / Oε2</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>Asp264 / Oδ1</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Asp264 / Oδ2</td>
<td>2.3</td>
</tr>
<tr>
<td>Mn(II)-PsaA&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>His67 / Nε2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>His139 / Nε2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Glu205 / Oε1</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Glu205 / Oε2</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Asp280 / Oδ1</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Asp280 / Oδ2</td>
<td>2.4</td>
</tr>
<tr>
<td>Zn(II)-PsaA&lt;sup&gt;c,e&lt;/sup&gt;</td>
<td>His67 / Nε2</td>
<td>2.0</td>
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<tr>
<td></td>
<td>Asp280 / Oδ2</td>
<td>2.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Numbering corresponds to the soluble, cysteine-less construct of MntC.  
<sup>b</sup> PDB: 4K3V  
<sup>c</sup> Numbering corresponds to the full-length PsaA.  
<sup>d</sup> PDB: 3ZTT  
<sup>e</sup> PDB: 1PSZ

1.4.2 Microbial Utilization of Mn(II)

Following cellular uptake, manganese becomes incorporated into biomolecules. Microbial enzymes that employ manganese as a cofactor contribute to defense against oxidative stress, nucleotide biosynthesis, primary metabolism, and antibiotic resistance (Figure 1.4.3). Recent investigations have focused on how host-mediated Mn(II) sequestration modulates oxidative killing as a result of Mn(II) chelation<sup>111-113</sup> and additional Mn(II)-dependent microbial processes warrant consideration in future work. The rest of this section discusses the role of Mn(II) superoxide dismutase and ribonucleotide reductase in pathogenesis, and considers the consequences of Mn(II)-sequestration by mammalian calprotectin (discussed in more detail in section 1.5.4) on bacterial Mn homeostasis.
Pathogens must survive the harsh chemical conditions of the immune response, including a host-mediated oxidative burst. Microorganisms therefore produce a number of detoxifying enzymes, including superoxide dismutases (SODs), to overcome oxidative stress. SODs catalyze the disproportionation of superoxide into oxygen and hydrogen peroxide, and require one of four metal cofactors: Fe, Mn, Ni, or Cu/Zn.\textsuperscript{14} The Fe, Mn, and Cu/Zn forms have been identified in human pathogens. Recent investigations addressing how the Mn(II)-sequestering protein calprotectin (CP, \textit{vide infra}) influences bacterial susceptibility to oxidative stress have focused on \textit{S. aureus} and a mouse model of \textit{S. aureus} infection.\textsuperscript{1,11-13} \textit{S. aureus} strains produce two SODs, SodA and SodM.\textsuperscript{15,16} SodA and SodM both exhibit a His\textsubscript{3}Asp primary coordination sphere and appear to require Mn as the redox-active cofactor; however, the identity of the cognate metal ion has not been rigorously determined for either enzyme, and some SODs are active with Mn or Fe.\textsuperscript{117-119} Given the expression of these two SODs, \textit{S. aureus} may be particularly susceptible to oxidative stress under Mn(II)-limiting conditions, providing an appropriate model organism to examine the effect of Mn(II) sequestration on SOD activity \textit{in vitro} and in an animal model of \textit{S. aureus} infection. Although not rigorously established to occur in \textit{S. aureus}, small-molecule Mn

\[
2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{Mn-SOD} \rightarrow \text{O}_2 + \text{H}_2\text{O}_2
\]
complexes also disproportionate superoxide and provide protection against oxidative stress in vivo.¹²⁰,¹²¹

Treatment of S. aureus Newman with CP in vitro reduces the total SOD activity of whole-cell lysates.¹¹¹ This effect was not observed upon mutation of sites 1 and 2 of CP, indicating that the transition metal-ion binding sites are necessary.¹¹¹ Moreover, the S. aureus SOD activity was restored upon addition of exogenous Mn(II) to the growth medium.¹¹¹ CP also increased the susceptibility of wild-type S. aureus to the superoxide-generating agent paraquat, and this effect was more pronounced for a ΔsodAΔsodM knockout mutant of Newman.¹¹¹ Thus, SodA and SodM contribute to the protection of S. aureus against superoxide, and CP decreases this effect.¹¹¹ Subsequent studies revealed that site 2 (His₆) was required to attenuate SOD activity of S. aureus.¹¹² Furthermore, the MntABC and MntH Mn(II)-uptake systems of S. aureus are required for full SOD activity in the presence of CP and for infection in a mouse model of the disease.¹¹³ Taken together, these results indicate that site 2 of CP competes with bacterial metal-ion transporters for Mn(II) and a consequence of CP-mediated Mn(II) sequestration is a reduction in staphylococcal SOD activity. Thus, although Mn(II) chelation is a bacteriostatic effect, CP may facilitate the bactericidal oxidative burst by reducing SOD activity. Despite these observations from in vitro studies, the ΔsodAΔsodM mutant of S. aureus was able to colonize both wild-type and CP knockout mice,¹¹¹ indicating that SODs may not be strictly required for pathogenesis and that other Mn-dependent processes are at work (vide infra).

Manganese-dependent SODs are found in many other microbial species and are considered to be virulence factors in many cases. The Lyme disease pathogen B. burgdorferi has no metabolic need for iron, and encodes a single Mn(II)-containing SOD for reactive oxygen species (ROS) detoxification.¹²²-¹²⁴ Other virulent species that utilize Mn-SOD include Neisseria gonorrhoeae,¹²⁰ Beauveria bassiana,¹²⁵ Streptococcus mutans,¹²⁶ Saccharomyces cerevisiae,¹²⁷ E. coli,¹²⁸,¹²⁹ H. influenzae,¹³⁰ and Treponema pallidum.¹³¹
Many pathogens also produce FeSOD and/or CuZnSOD. Whether CP influences the (mis)metallation and activity of these enzymes is currently unknown. Because different microbes employ different enzymatic arsenals to combat the host-mediated oxidative burst, the consequences of CP on the oxidative stress response will need to be assessed on a case-by-case basis.

Class I_{b} ribonucleotide reductase (RNR) is one compelling candidate for a Mn(II) enzyme that is disrupted by CP-dependent Mn(II) sequestration. RNRs are essential for life and responsible for the conversion of ribonucleotides to deoxyribonucleotides, the latter of which are the building blocks of DNA. Class I RNRs contain diiron (class I_{a}), dimanganese (class I_{b}), or dinuclear Fe/Mn (class I_{c}) metal cofactors. Many species of bacteria, including *E. coli*, *S. aureus*, and *Bacillus subtilis*, express class I_{b} RNRs, which are required for growth under iron-limited and/or aerobic conditions. Because iron is tightly bound by the host, class I_{b} RNRs may be particularly prevalent in pathogens and constitute a potential target for antibiotic development.

One example of a pathogenic bacteria that expresses class I_{b} RNR is *Streptococcus sanguinis*, a causative agent of infective endocarditis. A series of recent studies established that both Mn(II) and class I_{b} RNR are important for *S. sanguinis* pathogenicity. Deletion of the Mn(II) solute-binding protein SsaB (∆ssaB), which is homologous to PsaA and MntC, resulted in less cellular accumulation of Mn and Fe by *S. sanguinis* and attenuated its virulence in a rabbit model of infective endocarditis by >1,000-fold. Subsequently, a *S. sanguinis* ∆sodA mutant was found to be less virulent than wild-type by 10-100 fold. Taken together, these data showed that the ∆sodA mutant is more virulent than the ∆ssaB mutant, and suggested that other Mn-dependent processes contribute to heart valve colonization by *S. sanguinis*. The *S. sanguinis* genome encodes two forms of RNR, class I_{b} (nrdHEKF and nrdl) and class III (nrdD). Because NrdD is a strictly anaerobic form of RNR, the class I_{b} enzyme is required for aerobic growth of *S.
sanguinis. Biochemical studies of *S. sanguinis* class Ib RNR revealed that, like other class Ib RNRS, this enzyme may employ a dimanganese(III)-tyrosyl radical cofactor.\(^{139}\)

In order to evaluate the contribution of the class Ib RNR to *S. sanguinis* virulence, the same rabbit model of infective endocarditis was employed,\(^{140}\) and rabbits were infected with wild-type *S. sanguinis* or mutants lacking functional class Ib RNR.\(^{140}\) In contrast to the parent strain, which was recovered from heart tissue as expected, the \(\Delta_{\text{nr}d\text{HEKF}}\) and \(\Delta_{\text{nr}d\text{I}}\) mutant strains were unable to survive *in vivo*.\(^{140}\) Moreover, mutation of the class III RNR (\(\Delta_{\text{nr}d\text{D}}\)) had no effect on heart valve colonization.\(^{140}\) These results demonstrated that manganese enzymes other than Mn-SOD contribute to virulence, and revealed that disruption of RNR activity has a more pronounced effect on *S. sanguinis* viability *in vivo* than perturbation of its oxidative stress response. Given that deoxyribonucleotides are essential for life, reduction of RNR activity may be a general consequence of metal-ion withholding by the host. The effect of CP on cofactor assembly and the activity of class Ib RNR remains an avenue for future work.

The class Ic RNR, which contains a bimetallic Mn/Fe cofactor,\(^{143}\) is also of interest from the standpoint of Mn(II) sequestration by the host. The sexually transmitted intracellular pathogen *Chlamydia trachomatis* expresses a class Ic RNR with a Mn(IV)/Fe(III) cofactor that is necessary for activity *in vitro*,\(^{143,144}\) and it will be interesting to decipher whether Mn sequestration by the host perturbs its assembly. Despite the prevalence of RNRs in nature and the strict requirement of deoxyribonucleotide building blocks for the synthesis of DNA, some slow-growing pathogenic organisms, including *B. burgdorferi*,\(^{138}\) do not have genes encoding RNRs. Rather, these unusual microorganisms likely scavenge deoxyribonucleotides from the host.

There are many other enzymes that utilize Mn as a cofactor (Figure 1.4.3).\(^{1}\) For instance, *S. aureus* FosB is a Mn(II)-dependent enzyme that inactivates the antibiotic fosfomycin.\(^{145,146}\) Many enzymes involved in general metabolism, including *S. Typhimurium*
propionate kinase and the *E. coli* lactonase UlAG, are active with added Mn(II).

A potential mycobacterial virulence factor and putative oxidase named Rv0223 requires a Mn/Fe cofactor for activity. *Trichomonas vaginalis*, a sexually transmitted parasite, catalyzes the degradation of sphingomyelin, a component of the plasma membrane in the vaginal mucosa. Addition of Mn(II) to *T. vaginalis* cell extracts affords a two-fold increase in sphingomyelinase activity. It will be important to evaluate the contributions of these enzymes, and others, to virulence and the effect of CP on enzymatic activity. Lastly, the gut commensal *Lactobacillus* genus expresses a dinuclear Mn-containing catalase to detoxify hydrogen peroxide. The consequences of metal chelation by CP on the growth of commensal bacteria is largely unexplored; Mn(II) and Zn(II) sequestration may hinder beneficial flora in the inflamed gut.

1.5 Zinc and Manganese Sequestration by the Human Immune System

1.5.1 S100 Proteins: A Family of Metal-binding Proteins

S100 proteins are a family of Ca(II)-binding proteins. Examples of S100 proteins can be found across vertebrates, and S100 proteins are ubiquitous among mammals. Humans express at least 20 members of this protein family, and they play diverse roles in cell signaling, host defense, and disease progression (Table 1.5.1).

The first S100 proteins—later determined to be S100B and S100A—were isolated from the brain tissues of multiple mammals, and they earned the moniker "S100" because they were soluble in 100% ammonium sulfate. The family has since expanded to include at least 20 members in humans. In general, S100 proteins are characterized by being small (ca. 10-14 kDa), α-helical proteins that contain one or two Ca(II)-binding EF-hand domains. The C-terminal EF-hand resembles the EF-hands in calmodulin and is called the canonical EF-hand. The general sequence of the N-terminal EF-hand is specific to S100 proteins and is called the non-canonical EF-hand.
solution; however, higher-order oligomers of S100A7, S100A8/S100A9, S100A12, and S100B have been identified.\textsuperscript{157-160} S100 proteins exhibit many structural and functional similarities; however, current available studies indicate that they are expressed in a cell- and tissue-specific manner. In the human genome, the genes for the S100A proteins are clustered on the epidermal differentiation complex of chromosome 1.\textsuperscript{161}

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Localization</th>
<th>Proposed functions</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100A1</td>
<td>Cardiac tissue</td>
<td>Calcium cycling</td>
<td>162</td>
</tr>
<tr>
<td>S100A2</td>
<td>Kidney, heart, liver</td>
<td>Tumor suppression</td>
<td>163</td>
</tr>
<tr>
<td>S100A3</td>
<td>Hair cuticle</td>
<td>Structure and repair</td>
<td>164</td>
</tr>
<tr>
<td>S100A4</td>
<td>Keratinocytes</td>
<td>Pro-inflammatory</td>
<td>165</td>
</tr>
<tr>
<td>S100A5</td>
<td>Brain</td>
<td>Not determined</td>
<td>166</td>
</tr>
<tr>
<td>S100A6</td>
<td>Brain</td>
<td>Fibril formation</td>
<td>167</td>
</tr>
<tr>
<td>S100A7</td>
<td>Keratinocytes</td>
<td>Host-defense</td>
<td>168</td>
</tr>
<tr>
<td>S100A8</td>
<td>Granulocytes</td>
<td>Host-defense\textsuperscript{a}</td>
<td>169</td>
</tr>
<tr>
<td>S100A9</td>
<td>Granulocytes</td>
<td>Host-defense\textsuperscript{a}</td>
<td>169</td>
</tr>
<tr>
<td>S100A10</td>
<td>Broad range</td>
<td>Oncogenesis</td>
<td>171</td>
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<td>Epithelium</td>
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<td>Tumor marker</td>
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<td>Uterus</td>
<td>Ca(II) buffering</td>
<td>179</td>
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<tr>
<td>S100P</td>
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<td>Cancer cell growth</td>
<td>180</td>
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<tr>
<td>S100Z</td>
<td>Prostate, spleen</td>
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\textsuperscript{a}S100A8 and S100A9 contribute to host-defense as a heterooligomeric complex.

In addition to the Ca(II)-binding sites, many S100 proteins exhibit transition metal-ion binding properties and contain binding sites for the coordination of Zn(II), Cu(II), and Mn(II). These proteins include S100B, S100A7, S100A12, and the S100A8/S100A9 heterooligomer. In the following sections, these transition metal-ion binding S100 proteins will be discussed in additional detail.
EF-hand 1

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Figure 1.5.1 Sequence alignment of 20 human S100 proteins. The gene ID and the protein name are given. Residues that contribute to transition-metal-ion coordination in the sequences of S100A7, S100A8, S100A9, S100A12, and S100B are colored orange, and disulfide-bond forming cysteines in S100A3, S100A7, and S100A15 are colored green. The residues that comprise EF-hands 1 and 2 are indicated by the horizontal line.
1.5.2 S100B and S100A12

Human S100B and S100A12 are Ca(II), Zn(II), and Cu(II)-binding proteins. S100B is expressed by tissues of the nervous system,182 and S100A12 comprises about 5% of the cytosolic protein of neutrophils. The physiological roles of S100B and S100A12 are not well defined; however, the transition metal binding properties of these proteins have been investigated using biophysical techniques. S100B and S100A12 both coordinate Zn(II) and Cu(II); however, the physiological roles of divalent metal coordination are likely quite different.

S100B has been crystallized with Zn(II) and Ca(II), and two distinct Zn(II)-binding motifs are observed under different conditions (Figure 1.5.2).182 S100B coordinated Zn(II) with an interfacial His3Glu motif at pH 6.5 and pH 10.0. The ligand coordination geometry was tetrahedral with contributions from His16 and His26 from one monomer, and His86 and Glu90 from the second monomer. At pH 9.0, Glu89 was replaced with His91, and Zn(II) was coordinated in an unusual His4 motif. The physiological role Zn(II) coordination by S100B is currently unknown. S100B also coordinates copper, and characterization of the Cu(II)-bound form of S100B by electron paramagnetic resonance and optical absorption spectroscopy are indicative of a Type 2 copper center.183 It is likely that Zn(II) and Cu(II) share the same binding site; however, the coordination motif of S100B has not been rigorously determined. The Cu(II)-bound form exhibits weak catecholase activity, indicating Cu-bound S100B may cycle between Cu(II) and Cu(I) oxidation states under certain conditions.183 Copper binding by S100B may suppress copper-induced oxidative damage and cell damage.178,184,185 Moreover, S100B interacts with the dopamine D2 receptor, and it is unclear how metal coordination may influence this interaction.186
Figure 1.5.2. X-ray crystal structures of the Ca(II)- and Zn(II)-bound forms of S100B at pH 10.0 (A,B) and pH 9.0 (C,D). (A) The S100B dimer crystallized at pH 10.0 (PDB 3D10). S100B is shown in teal, and metal-binding residues are shown as orange sticks. Ca(II) ions are shown as yellow spheres, and Zn(II) ions are shown as chocolate spheres. (B) The His86-Glu90 Zn(II)-binding motif of S100B at pH 10.0 is formed by His16 and His26 from one subunit, and His86 and Glu90 from the second subunit. (C) The S100B dimer crystallized at pH 9.0 (PDB 3CZT). The His86-His91 Zn(II)-binding motif of S100B at pH 9.0 is formed by His16 and His26 from one subunit, and His86 and His91 from the second subunit.

S100A12 is also a Cu(II)- and Zn(II)-binding S100 protein, and the crystal structure of the Cu(II)- and Ca(II)-bound form is shown in Figure 1.5.3. S100A12 is expressed by granulocytes and modulates inflammation by interacting the with the receptor for advanced glycation end products (RAGE). S100A12 has been crystallized in apo, Zn(II)-
bound, Ca(II)-bound, and Cu(II)- and Ca(II)-bound forms. S100A12 coordinates Cu(II) and Zn(II) at an interfacial His\textsubscript{3}Asp motif. This site is formed by His\textsubscript{16} and Asp\textsubscript{26} from one monomer, and His\textsubscript{86} and His\textsubscript{90} from the second monomer (Figure 1.5.3). The oligomerization properties of S100A12 are complex: S100A12 may form dimers, tetramers, or hexamers depending on the metals that are present.

**Figure 1.5.3.** X-ray crystal structure of Cu(II)- and Ca(II)-bound S100A12 (PDB ID: 10DB). (A) The S100A12 dimer with Cu(II) and (B) an expansion of the Cu(II)-binding site. (C) The asymmetric unit contains three S100A12 dimers and may represent an approximation of the S100A12 hexamer. Cu(II) ions are shown as teal spheres, and Ca(II) ions are shown as yellow spheres.

1.5.3 S100A7 and S100A15

S100A7 and S100A15 are both Ca(II)- and Zn(II)-binding proteins that are expressed by keratinocytes. These proteins are very closely related and exhibit 94% amino acid sequence identity. The genes encoding these proteins were initially annotated as s100a7a (S100A15) and s100a7c (S100A7), and have only been given different designations in more recent literature. S100A7, also called psoriasin, is expressed in high levels in psoriatic skin lesions where it contributes to a general lack of microbial flora. S100A7 was isolated from human skin and identified as an *E.coli*-icidal component of the skin proteome following
HPLC purification. The antibacterial activity of S100A7 is attenuated by excess Zn(II), and it was proposed that S100A7 kills *E. coli* through zinc sequestration. S100A15 also has a proposed, yet ill-defined, role in host-defense; the potential mechanism of action is unclear. In addition to their putative antimicrobial properties, S100A7 and S100A15 expression in the cell cytoplasm is associated with multiple cancer pathologies. Further studies are required to elucidate how S100A7 and S100A15 may disrupt normal cellular metabolism. A significant challenge to elucidating the mechanisms of action of these proteins is the lack of a suitable mouse homolog. Normal mice do not exhibit psoriasis, and the mouse ortholog of S100A15 exhibits relatively low homology to its human counterparts (sequence alignments are presented in Appendix 1). Thus, there is no suitable animal model available to study the roles of these proteins *in vivo*.

S100A7 and S100A15 are 101-residue, 11.3 kDa polypeptides and form obligate homodimers in solution. Like other S100 proteins, S100A7 and S100A15 each contain one canonical EF-hand domain in the C-terminal region. This C-terminal EF-hand has been crystallized with either Ca(II) or Ho(III). The non-canonical N-terminal EF-hand, observed in related S100 proteins, has a truncated Ca(II)-binding loop and does not appear to be able to coordinate Ca(II) or other cations at this position. Early studies indicated that S100A7 did not coordinate Zn(II) tightly. Nevertheless, the crystal structure of Zn(II)- and Ca(II)-bound S100A7 reveals an interfacial His$_3$Asp motif that is formed by His18 and Asp25 from one monomer, and His87 and His91 from the second polypeptide (Figure 1.5.4). This primary coordination motif is identical to the Zn(II)-binding sites in S100A12 and CP (site 1, *vide infra*). S100A15 has a glycine residue at position 25, and does not house a His$_3$Asp motif. Nevertheless, S100A15 has been crystallized with zinc(II) at the interfacial His$_3$ site with Cl$^-$ completing a tetrahedral coordination environment.
The zinc-binding properties of S100A7 and S100A15 are not well understood, and the affinities of these proteins for zinc have not been established. Bacterial zinc solute-binding proteins typically have apparent $K_d$ values on the order of $\leq 10^{-8}$, we contend that S100A7 must have a comparable affinity in order to compete with these transporters.

1.5.4 Calprotectin

CP is a heterooligomer of S100A8 (also called calgranulin A or MRP8) and S100A9 (also called calgranulin B or MRP14). CP was initially identified as an abundant component of human neutrophils. Early work refers to CP as leukocyte protein L1, and CP was identified as a marker of inflammation in cystic fibrosis patients as “cystic fibrosis protein” or “cystic fibrosis antigen”, as well as other pathologies. Subsequent studies revealed that CP exhibits in vitro antimicrobial activity, and that this activity is abrogated upon addition of exogenous zinc. Moreover, CP is associated with the zinc homeostasis disorder hyperzincaemia. Taken together, these studies provided evidence that CP functions as a high-affinity zinc chelator. A mouse model of S. aureus infection later demonstrated that CP exhibits antibacterial activity through sequestration of Zn(II) and...
Mn(II). CP is therefore the first, and to date only, identified mammalian Mn(II)-sequestering protein.

Structurally, CP is a heterodimer or heterotetramer of S100A8 (α) and S100A9 (β). Although S100A8 and S100A9 may form homodimers (Figure 1.5.4), these S100 proteins preferentially form heterodimers in vitro. S100A8 is a 93 amino acid, 10.8 kDa polypeptide. S100A9 is the largest member of the human S100 protein family with 114 amino acids and is 13.2 kDa. The addition of excess Ca(II) to CP (αβ) induces the formation α2β2 tetramers. The CP heterotetramer has been crystallized in the presence of Ca(II) (see Chapters 2-4).

Figure 1.5.4. Crystal structures of the human S100A8 (A and B) and S100A9 (C) homodimers. (A) The S100A8 homodimer and (B) an interfacial His4 motif (PDB ID: 1MR8). The A8 homodimer has not been identified in vivo, and it is unknown if S100A8 plays a role in mammalian transition metal-ion homeostasis. (B) An expansion of a putative His4 transition metal-ion binding motif of S100A8. Residues His17, His27, His83, and His87 are shown as orange sticks. (C) The S100A9 homodimer (PDB ID: 1IRJ). Residues 87-114 of the C-terminal region of S100A9 are disordered in the available crystal structure. Residues His20 and Asp30, which contribute to Zn(II) and Mn(II) binding in CP, are shown as orange sticks. Ca(II) ions are shown as yellow spheres.

Alignment of S100A8 and S100A9 with S100A7, S100A15, and S100B reveals that the following interfacial transition metal-ion binding residues are conserved: (A8)His17, (A8)His27, (A8)His83, (A8)His87, (A9)His20, (A9)Asp30, (A9)His91, and (A9)His95. The C-
terminal region of S100A9 is extended relative to other S100 proteins and contains several moieties that are capable of transition-metal binding. Subsequent studies have revealed that (A9)His103 and (A9)His105 contribute to the sequestration of Mn(II) by CP, and these experiments will be discussed in Chapters 3 and 4 as well as Appendix 4.112,222,223

Figure 1.5.6. X-ray crystal structure of human CP (αβ₂) (PDB ID: 1XK4).157 The recombinant protein employed in the crystallization was CP-Ser and it crystallized as the heterotetramer in the presence of Ca(II). S100A8 (α) is depicted in green and S100A9 (β) is shown in blue. The Ca(II) ions are represented as yellow spheres. The S100A9 C-terminal tail (residues 92-114 and 96-114 of each A9 subunit) was disordered and is not shown. (A) The αβ₂ heterotetramer. (B) One heterodimeric unit of the structure presented in A is shown in a different orientation to show the putative His₃Asp site (site 1) and His₄ (site 2) metal-binding sites. This depiction includes the Ca(II) ions and is used as a model for the apo heterodimer in this work. S100A8 and S100A9 each contribute two residues to each metal-binding site. (C) Close-up view of the His₃Asp site. (D) Close-up view of the His₄ site.

1.6 Thesis Overview

This thesis details our efforts to elucidate the metal-binding properties of the human host-defense proteins calprotectin and S100A7. Chapter 2 describes the Co(II)- and Zn(II)-binding properties of CP, as well as the discovery that CP responds to Ca(II)-ion gradients to modulate its Zn(II) affinity and antibacterial activity. Chapter 3 and 4 describe spectroscopic and biophysical experiments that identify and characterize the unique hexahistidine site that is required for Mn(II)-sequestration. Together, these studies provide a
foundation for future work with these transition-metal ion sequestering proteins and provide new insights into metal-ion sequestration by the mammalian innate immune system.
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Chapter 2: Calcium Ion Gradients Modulate the Zinc(II) Affinity and Antibacterial Activity of Human Calprotectin

This chapter is adapted from J. Am. Chem. Soc. 2012, 134, 18089-18100.
2.1 Introduction

Transition metal ions are essential nutrients that are required for all forms of life. Invading pathogens must acquire transition metals such as Mn(II), Fe(II)/Fe(III), and Zn(II) from the host in order to establish an infective lifestyle. Metal-ion withholding, also called nutritional immunity, is therefore an important facet of the mammalian innate immune response. The human proteins transferrin, lactoferrin, and siderocalin prevent bacterial uptake of Fe(III) and provide a canonical example of nutritional immunity. Zinc and manganese are also important transition metal ions at the host-pathogen interface, and are required for pathogenicity of many microorganisms.

The human protein calprotectin (CP) has been identified as a key component of the human innate immune system that sequesters Zn(II) and Mn(II) from invading pathogens. CP is a heterodimer of S100A8 (93 amino acids, 10.8 kDa, subunit) and S100A9 (114 amino acids, 13.2 kDa, β subunit) (Figure 2.1.1). CP is expressed in large quantities in white blood cells called neutrophils, where it comprises ca. 40% of the cytosolic protein. These white blood cells are rapidly recruited to sites of infection where they release a potent cocktail of antimicrobial agents. CP has been found at sites of infection in concentrations up to and in excess of 1 mg/mL, and CP is a component of neutrophil extracellular traps (NETs). CP inhibits the growth of a diverse array of microbes in vitro, including Candida albicans, Escherichia coli, Salmonella enterica serovar Typhimurium, Staphylococcus aureus, and Borrelia burgdorferi.

Seminal investigations established that the growth inhibitory properties of CP are reversed upon the addition of excess Zn(II). Moreover, S. Typhimurium expresses the high-affinity Zn(II)-uptake system ZnuABC to overcome CP in the inflamed gut. In a model of S. aureus infection, CP inhibited colonization by sequestering Mn(II) and enhanced the susceptibility of S. aureus to oxidative stress by inhibiting Mn(II)-dependent
disproportionation of superoxide. Taken together, these studies support the notion that CP binds to Zn(II) and/or Mn(II) with high affinity and sequesters these essential metal ions. A molecular level understanding of the metal-binding properties of CP was largely unexplored prior to the work presented in this chapter and required further elucidation.

CP is a heterooligomer of S100 proteins, and each subunit contains Ca(II)-binding EF-hand domains. Ca(II)-binding induces the formation of $\alpha_2\beta_2$ heterotetramers; however, the physiological role of the tetramer, as well as the effect of Ca(II)-ion binding on the Zn(II) and Mn(II)-binding properties of CP, remain poorly understood. A crystal structure of the Ca(II)-bound CP tetramer was reported in 2007, and revealed two putative transition metal-ion binding motifs at the S100A8/S100A9 dimer interface. Site 1 is a His$_3$Asp motif comprised of residues His83 and His87 from S100A8, and residues His20 and Asp30 from S100A9. Site 2 is a relatively unusual His$_4$ site that is formed by residues His17 and His27 from S100A8 and residues His91 and His95 from S100A9. Subsequent crystallographic and biochemical investigations established that (A9)His103 and (A9)His105 also contribute to metal-binding at site 2 to form an unprecedented His$_6$ site. These studies are presented in Chapter 4 and Appendix 4.

This chapter describes solution spectroscopic and thermodynamic investigations of the Zn(II)-binding properties of human calprotectin. Using Co(II) as a spectroscopic probe, we have identified spectroscopic signatures for metal-ion binding to human calprotectin. We have also evaluated the Zn(II) affinity under conditions of high and low Ca(II) and conclude that CP employs Ca(II) ion gradients to tune its affinity for Zn(II) as well as its antibacterial activity.
Figure 2.1.1. Proposed antimicrobial mechanism and structural features of human CP. (A) Proposed mechanism of action. CP is released from the neutrophil into the extracellular milieu where it competes with bacterial metal-ion transporters for bioavailable zinc(II) and manganese(II). (B,C) The two putative transition metal-binding sites of human CP revealed at the dimer interface by X-ray crystallography (PDB ID: 1XK4). S100A8 is colored green and S100A9 is colored blue. The putative metal-binding residues are colored orange. Panel B shows the His3 Asp site (site 1) and panel C depicts the His site (site 2). (D) Amino acid sequence alignment of the human calprotectin subunits S100A8 and S100A9 with human S100A7 and human S100A12. Alpha-helices I-IV, the Ca(II)-binding loops, and the linker regions of S100A8 and S100A9 are indicated above the alignments. The N-terminal EF-hand of S100A7 is truncated and does not appear to coordinate Ca(II). The identified (S100A7, S100A12) and putative (S100A8, S100A9) metal-binding residues are colored orange. The cysteine residues that were mutated to serine in this work are colored red. Both S100A7 and S100A12 form homodimers, coordinate transition metals at interfacial His3 Asp sites, and are involved in the innate immune response.
2.2 Experimental

2.2.1 Materials and General Methods

All solvents, reagents, and chemicals were obtained from commercial suppliers and used as received unless otherwise noted. FluoZin-3 (FZ3) and Mag-Fura-2 (MF2) were purchased from Invitrogen and Zinpyr-4 (ZP4) was obtained from Strem Chemicals, Inc. Zincon monosodium salt was obtained from Alfa Aesar. Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. and used as received (standard desalting protocol). A Biorad MyCycler thermocycler was employed for all polymerase chain reactions (PCR). Chemically competent *E. coli* TOP and BL21(DE3) cells were prepared in house by standard protocols. An illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) were utilized for purifying all PCR products, and a Qiagen miniprep kit was employed for all plasmid isolations. DNA sequencing was performed by staff members at the MIT Biopolymers facility. All buffers, aqueous solutions, and oligonucleotide stock solutions were prepared using Milli-Q water (18.2 mΩ-cm, 0.22-µm filter). Milli-Q water was also used to prepare Luria broth (LB), LB agar plates, and Tryptic Soy broth (TSB).

An Agilent 8453 diode array spectrophotometer was employed for measuring OD$_{600}$ values of bacterial cultures unless otherwise noted. Protein concentrations were routinely determined by using a calculated extinction coefficient ($\varepsilon_{280} = 18,450 \text{ M}^{-1} \text{ cm}^{-1}$), and the absorption readings were taken with either a BioTek Synergy HT plate reader outfitted with a calibrated Take3 Micro-Volume plate or an Agilent 8453 diode array spectrophotometer using a 1-cm path length cuvette (Starna). All CP concentrations are in terms of $\alpha\beta$ dimer, including in the presence of Ca(II). All reported stoichiometries are per CP heterodimer ($\alpha\beta$).
2.2.2 General Methods for Optical Absorption and Fluorescence Spectroscopic Measurements

All precautions were taken to minimize metal-buffer equilibria and metal-ion contaminations. Non-coordinating buffers were employed for metal-binding studies and dissociation constant determination to prevent complications resulting from metal-buffer equilibria. Aqueous solutions were prepared with fresh Milli-Q (18.2 MΩ, 0.22-µm filter) water. Metal-free TraceSELECT (99.999%) NaCl was purchased from Sigma-Aldrich, and metal-free ULTROL grade HEPES (free-acid) was obtained from Calbiochem. To minimize metal-ion contaminations, only Teflon-coated spatulas were employed to transfer all buffer reagents, and the solutions were treated with 10 g/L of Chelex resin by stirring in a polypropylene beaker for at least 1 h prior to use. Chelex was removed by filtering the mixture through a 0.22-µm filter (Corning) or by centrifugation. Some fluorescent sensors detected Ca(II) contamination from the 0.22-µm filters, and centrifugation was employed for removing Chelex from buffer employed for fluorescence spectroscopy. The buffers were stored in sterile polypropylene tubes.

Protein aliquots were thawed on ice, and the storage buffer was exchanged (at least three 5x dilutions) by using a 0.5-mL Amicon centrifugal filter device with a 10-kDa MWCO membrane (Millipore). Cobalt stock solutions were prepared from 99.999% CoCl₂ hydrate (Sigma Aldrich) and Milli-Q water. All zinc stock solutions were prepared from 99.999% anhydrous ZnCl₂ (Sigma Aldrich) and Milli-Q water. All Co(II)-binding studies were conducted at pH 7.0 in 75 mM HEPES, 100 mM NaCl buffer. The Co(II) studies were conducted at pH 7.0 because CP-Ser (400 µM) precipitated following addition of Co(II) at pH 7.5. With the exception of Co(II) displacement experiments, all Zn(II)-binding studies were conducted at pH 7.5 in 75 mM HEPES, 100 mM NaCl buffer. DMSO stock solutions of approximately 1 mM ZP4 were prepared, partitioned into 50-µL aliquots, stored at -20 °C, and thawed immediately before use. Stock solutions of FZ3 and MF2 were prepared in Milli-
Q water, partitioned into aliquots, and stored at -20 °C. These metal- and light-sensitive chromophores were covered in aluminum foil, thawed immediately before use, and handled in the dark. Stock solutions of Zincon in DMSO were prepared immediately before use and kept covered in aluminum foil.

Optical absorption spectra were collected on a Beckman Coulter DU 800 spectrophotometer thermostatted at 25 °C with a Peltier temperature controller or an Agilent 8453 diode-array spectrophotometer controlled with manufacturer-supplied software and thermostatted at 25 °C by a circulating water bath. Quartz cuvettes with 1-cm path lengths (Starna) were employed for all optical absorption measurements. All optical and fluorescence spectroscopic measurements were performed at least two times, and all titrations were performed at least in triplicate. Fluorescence spectra were collected on a Photon Technologies International QuantaMaster 40 fluorimeter outfitted with a continuous xenon source for excitation, autocalibrated QuadraScopic monochromators, a multimode PMT detector, and a circulating water bath maintained at 25 °C. This instrument was controlled by the FelixGX software package. FelixGX was routinely employed to integrate the emission spectra.

2.2.3 Cloning and Mutagenesis of S100A8 and S100A9 Genes

Synthetic genes containing the E. coli-optimized nucleotide sequences for human S100A8 and S100A9 were obtained in the pJ201 vector from DNA 2.0. Both synthetic genes were designed to include the N-terminal TEV protease cleavage site (ENLYFQG) at the 5’ end of the start codon to provide TEV-s100A8 and TEV-s100A9; however, the TEV cleavage site was not utilized in this work. The corresponding nucleotide and amino acid sequences are given below:
**E. coli optimized nucleotide sequence for TEV-S100A8:**

GAGAATCTGTATTCCAGGGTATGCTGACCGAGCTGGAGAAAGCGCTGAACTCCATTA
TCGACGCTTTACCACAAATACAGCCTGATCAAGGGTTAACCTCCCGCTCTAATCGTGA
GATCTGAAGAAATTTGCTGAAACCCCAAAGTGCAGTACATCCGCAAAAAGGGCGCAG
ACGTGTGGTTAAGGAAGTGGACATTTACACGGGTGCGCTGTGAATTTCCAAGAATT
CTGATTCTGGTCATCAAATGGGTGTTGCGGCCCATATAAAAGAGAGAGAGAGCG
ATAAAGAG

**Translated sequence for TEV-S100A8:**

**ENLYFQ** GM LEKALNSIIDVYHKLYSLIKGNFHAYRYDDLKKL
LETECPQYIRKKGADVVFKELDINTDGAVNFEFLILVIKMVA
AHKKSHESHKE

**E. coli optimized nucleotide sequence for TEV-S100A9:**

GAGAATTGTTACTTCCAAGGATGACCTGCAAGATGAGCCAGCTGGGAACGCATAATCG
AAACCATTATAACCTTCCACCAATACTCCGTCAATGTGTCATCGGCACACGCTG
AACCGAGGCGAGTTAAGGACGCTGTCGTAAGATCTGCAAGACTTCCTGAAAGAGG
AGAAACAAGAGAAGGAGTGGAGGACTGCTGACACATGCCAAAAATCG
CAAGAAGGAGATGACCGTGCTGCTGGGGCCTGACGTGGGCAGGACG
CAGCAGAAATGCTGACGGTGATGAGGCTGGCCCGCACCATCACAAACCGGCTG
GGCGAAGGTACTCCG

**Translated sequence for TEV-S100A9:**

**ENLYFQ** GMLTELEKALNSIIDVYHKLYSLIKGNFHAYRYDDLKKL
LETECPQYIRKKGADVVFKELDINTDGAVNFEFLILVIKMVA
AHKKSHESHKE

**Translated sequence for TEV-S100A9:**

**ENLYFQ** GMTCKMSQLERNIETFHTHYSVKLGHDTPNLQGEF
KELVRLQLNFFLLKKNKNEKVKHIEMDLNTADKQLSFEFIM
LMARLTWASHEKMHGDEGPQHGHKPGGLGEGTP

82
The plasmids pJ201-TEV-S100A8 and pJ201-TEV-S100A9 were each dissolved in 2 μL of Milli-Q water and transformed (2 μL) into chemically-competent E. coli TOP10 cells for plasmid amplification. Single colonies were selected and grown in 5 mL of LB media at 37 °C for ca. 16 h. The plasmids were isolated from the overnight cultures by using a miniprep kit (Qiagen) and verified by DNA sequencing. The S100A8 and S100A9 genes were subcloned into pET41a without the TEV cleavage site to give pET41a-S100A8 and pET41a-S100A9. The amplification primers are given in Tables 2.2.1 and 2.2.2. Polymerase chain reactions (PCR) were performed with PfuTurbo DNA polymerase (Stratagene) and the amplified gene sequences were purified using an illustra GFX kit. The PCR products and the pET41a vector were digested with Ndel and Xhol (New England Biolabs), ligated by using T4 DNA ligase (New England Biolabs), and transformed into chemically-competent E. coli TOP10 cells. Single colonies were selected to inoculate 5 mL of LB media containing 50 μg/mL of kanamycin and the cultures were grown for ca. 16 h. A miniprep kit was used to isolate the plasmids, and the identity of pET41a-S100A8 and pET41a-S100A9 were verified by DNA sequencing (MIT Biopolymers).

A modified Quick-Change site-directed mutagenesis protocol (Stratagene) was employed to generate the S100A8 and S100A9 mutants. The templates, primers, and primer pairings are listed in Tables 2.2.1-2.2.3. The PCR protocol used for all mutagenesis reactions was: 95 °C for 30 min (1x); 55 °C for 1 min, 68 °C for 17 min (25x); 4 °C hold. Following PCR amplification using PfuTurbo DNA polymerase, the template plasmid was digested with DpnI (New England Biolabs; 2 μL added to a 50 μL PCR reaction in two 1-μL aliquots at t = 0 and t = 1.5 h) for 3 h at 37 °C. The digests were transformed into chemically-competent E. coli TOP10 cells. Overnight cultures (5 mL, 50 μg/mL kanamycin) were grown from single colonies and the purified plasmids were obtained by using a miniprep kit. The DNA sequences and presence of the desired mutation(s) were verified by DNA sequencing.
**Table 2.2.1.** Primers Employed for Sub-Cloning and Site-Directed Mutagenesis of S100A8. 

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8-1</td>
<td>5'-GGAATTCCATATGCTGACGAGCTGG-3'</td>
</tr>
<tr>
<td>A8-2</td>
<td>5'-GATCCCTCGAGTTACTCTTTATGGCTCTTCTCG-3'</td>
</tr>
<tr>
<td>C42S-1</td>
<td>5'-CTGGAAACCGAAAGCCCCGAGTTACATCC-3'</td>
</tr>
<tr>
<td>C42S-2</td>
<td>5'-GACCTTTGGCTTTCGGGCGTGATGG-3'</td>
</tr>
<tr>
<td>H17A-1</td>
<td>5'-CGACGTTTACGCGAAATACAGCCTG-3'</td>
</tr>
<tr>
<td>H17A-2</td>
<td>5'-GCTGCAAATGCGCTTTATGTCGGAC-3'</td>
</tr>
<tr>
<td>H27A-1</td>
<td>5'-CAAGGGGTAACTTTGCGCGGTCTATCGT-G-3'</td>
</tr>
<tr>
<td>H27A-2</td>
<td>5'-GTCCCATTTGAAACGCGCCAGATAGCAC-3'</td>
</tr>
<tr>
<td>H83A-1</td>
<td>5'-CAAATGGGTGGGTGGGCTCCGGCGGaGAAGAGGCACCGAAAG-3'</td>
</tr>
<tr>
<td>H83A-2</td>
<td>5'-GTTTTACCAACAGGCCGGGGCTTTCTCTCGGTGCTTC-3'</td>
</tr>
<tr>
<td>H87A-1</td>
<td>5'-GCCCATAGAAGAGCCGGAGAAGAGGAGCCATAAG-3'</td>
</tr>
<tr>
<td>H87A-2</td>
<td>5'-CGGTATTCTTCTGCGGGCTCTTCTCTCGGTATCC-3'</td>
</tr>
<tr>
<td>(H83A)-H87A-1</td>
<td>5'-GCGCGGAAGAAGAGGCCTCCGGGAAGAGGAGCCATAAG-3'</td>
</tr>
<tr>
<td>(H83A)-H87A-2</td>
<td>5'-CGGGCGCTTTCTCTCGGGCTTTCTCTCGGTATCC-3'</td>
</tr>
</tbody>
</table>

* Underlined black codons indicate restriction sites. Bold codons indicate stop codons. The H83A mutation in the (H83A)-H87A primers is italicized and highlighted in blue. The codons containing the mutations are underlined and highlighted in red.
Table 2.2.2. Primers Employed for Sub-Cloning and Site-Directed Mutagenesis of S100A9.\(^{a}\)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A9-1</td>
<td>5'-GGAATTCCATATGACCTGCAAGATGA-3'</td>
</tr>
<tr>
<td>A9-2</td>
<td>5'-GATCCTCGAGTTACGGAGTGACTCTCGGCAGGA-3'</td>
</tr>
<tr>
<td>C3S-1</td>
<td>5'-CAAGGCATGACCAGCAAGATGAGCCAGC-3'</td>
</tr>
<tr>
<td>C3S-2</td>
<td>5'-GTTCCGTACTGCTCGTCTACTCGGTCG-3'</td>
</tr>
<tr>
<td>H20A-1</td>
<td>5'-CATTATCAATACCTCGGCAATCTCCGTCGAATTG-3'</td>
</tr>
<tr>
<td>H20A-2</td>
<td>5'-GTAAATAGTTATGGAAGCGGCTTTGAGCCATTAAAC-3'</td>
</tr>
<tr>
<td>D30A-1</td>
<td>5'-CAATTGGGTCATCCGCGACCGCTGCTGCTTGGATGACCAGGCGAG-3'</td>
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<tr>
<td>D30A-2</td>
<td>5'-GTTTACCCAGTGACCAGCCTGCTGCTTGGATGACCAGGCGAG-3'</td>
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<tr>
<td>H91A-1</td>
<td>5'-GCTCGACGTGCGAGCGGAGACCTGAAGGTAAGGGAAGG-3'</td>
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<tr>
<td>H91A-2</td>
<td>5'-CAGACTGCACCCACGTCCGCTGTCTTATGATCTCC-3'</td>
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<tr>
<td>H95A-1</td>
<td>5'-GAGCCACGAGAAATGGCGGAAGGTAGTGAAGGTCC-3'</td>
</tr>
<tr>
<td>H95A-2</td>
<td>5'-CTCGGTGCTCTTTAACCCTCCCTCCTACACTCCAGG-3'</td>
</tr>
<tr>
<td>(H91A)-H95A-1</td>
<td>5'-GAGCGCGAAGAAATGGCGGAAGGTAGTGAAGGTCC-3'</td>
</tr>
<tr>
<td>(H91A)-H95A-2</td>
<td>5'-CTCGCGCTCTTTAACCCTCCCTCCTACACTCCAGG-3'</td>
</tr>
</tbody>
</table>

\(^{a}\) Underlined black codons indicate restriction sites. Bold codons indicate stop codons. The H91A mutation in the (H91A)-H95A primer is italicized and highlighted in blue. The codons containing the mutations are underlined and highlighted in red.
### Table 2.2.3. Templates and Primer Pairings Employed in Site-Directed Mutagenesis.

<table>
<thead>
<tr>
<th>Template</th>
<th>Product</th>
<th>Primer Pairing</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET41a-S100A8</td>
<td>pET41a-S100A8(C42S)</td>
<td>C42S-1, C42S-2</td>
</tr>
<tr>
<td>pET41a-S100A8(C42S)</td>
<td>pET41a-S100A8(C42S)(H17A)</td>
<td>H17A-1, H17A-2</td>
</tr>
<tr>
<td>pET41a-S100A8(C42S)</td>
<td>pET41a-S100A8(C42S)(H27A)</td>
<td>H27A-1, H27A-2</td>
</tr>
<tr>
<td>pET41a-S100A8(C42S)</td>
<td>pET41a-S100A8(C42S)(H83A)</td>
<td>H83A-1, H83A-2</td>
</tr>
<tr>
<td>pET41a-S100A8(C42S)</td>
<td>pET41a-S100A8(C42S)(H87A)</td>
<td>(H83A)-H87A-1, (H83A)-H87A-2</td>
</tr>
<tr>
<td>pET41a-S100A8(C42S)(H17A)</td>
<td>pET41a-S100A8(C42S)(H17A)(H27A)</td>
<td>H27A-1, H27A-2</td>
</tr>
<tr>
<td>pET41a-S100A8(C42S)(H83A)(H87A)</td>
<td>pET41a-S100A8(C42S)(H83A)(H87A)</td>
<td>(H83A)-H87A-1, (H83A)-H87A-2</td>
</tr>
<tr>
<td>pET41a-S100A8(C42S)(H87A)</td>
<td>pET41a-S100A8(C42S)(H87A)</td>
<td>(H83A)-H87A-1, (H83A)-H87A-2</td>
</tr>
<tr>
<td>pET41a-S100A8(C42S)</td>
<td>pET41a-S100A8(C42S)(H87A)</td>
<td>(H83A)-H87A-1, (H83A)-H87A-2</td>
</tr>
<tr>
<td>pET41a-S100A9(C3S)</td>
<td>pET41a-S100A9(C3S)(H20A)</td>
<td>H20A-1, H20A-2</td>
</tr>
<tr>
<td>pET41a-S100A9(C3S)</td>
<td>pET41a-S100A9(C3S)(D30A)</td>
<td>D30A-1, D30A-2</td>
</tr>
<tr>
<td>pET41a-S100A9(C3S)</td>
<td>pET41a-S100A9(C3S)(H91A)</td>
<td>H91A-1, H91A-2</td>
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<td>pET41a-S100A9(C3S)(H91A)</td>
<td>pET41a-S100A9(C3S)(H95A)</td>
<td>H95A-1, H95A-2</td>
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<td>pET41a-S100A9(C3S)(H20A)(D30A)</td>
<td>pET41a-S100A9(C3S)(H20A)(D30A)</td>
<td>D30A-1, D30A-2</td>
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<tr>
<td>pET41a-S100A9(C3S)(H91A)(H95A)</td>
<td>pET41a-S100A9(C3S)(H91A)(H95A)</td>
<td>(H91A)-H95A-1, (H91A)-H95A-2</td>
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<td>pET41a-S100A9(C3S)(H20A)(D30A)(H91A)</td>
<td>pET41a-S100A9(C3S)(H20A)(D30A)(H91A)</td>
<td>(H91A)-H95A-1, (H91A)-H95A-2</td>
</tr>
<tr>
<td>pET41a-S100A9(C3S)</td>
<td>pET41a-S100A9(C3S)(H20A)(D30A)(H91A)</td>
<td>(H91A)-H95A-1, (H91A)-H95A-2</td>
</tr>
</tbody>
</table>

### 2.2.4 Overexpression and Purification of CP and Mutants

The pET41a-S100A8 and pET41a-S100A9 overexpression plasmids were transformed into chemically-competent *E. coli* BL21(DE3) cells. Cultures from single colonies were grown to saturation in LB media containing 50 μg/mL of kanamycin, and freezer stocks were prepared by diluting the overnight cultures with an equal volume of sterile 1:1 water/glycerol, flash frozen in liquid nitrogen, and stored at -80 °C. For protein overexpression, overnight cultures were grown to saturation in LB media containing 50 μg/mL of kanamycin (37 °C, 150 or 175 rpm, t = 16 h) and diluted 1:100 into fresh LB media containing 50 μg/mL of kanamycin and incubated at 37 °C with shaking (150 or 175 rpm).
The bacterial cultures were monitored by measuring the turbidity at 600 nm, and overexpression was induced with 500 µM of IPTG at OD₆₀₀ ≈ 0.6-0.8. The cultures were incubated at 37 °C for an additional 3-4 h (OD₆₀₀ ≈ 1.5), and pelleted by centrifugation (4200 rpm, 30 min, 4 °C). The cell pellets were transferred to pre-weighted 50-mL polypropylene centrifuge tubes, flash frozen in liquid nitrogen, and stored at -80 °C. These cultures were routinely grown in multiple 1- or 2-L portions in 2- or 4-L baffled flasks and stored as 1- or 2-L pellets. Approximately 2-3 g / L of *E. coli* cells (wet weight) were obtained from each preparation, and the cell pellets were stored at -80 °C for up to three months.

The purification of the heterodimeric CP from its constituent polypeptides was performed by modifying published protocols. All buffers were stored at 4 °C, and all purification steps were performed on ice or in a cold room at 4 °C. In a typical purification, cell pellets for S100A8 and S100A9 were thawed on ice. Each pellet was resuspended in ca. 30 mL of lysis buffer A (50 mM Tris, 100 mM NaCl, 10 mM BME, 1 mM EDTA, 0.5% Triton-X100, pH 8.0), and a 300-µL aliquot of 100 mM PMSF (EtOH, stored at -20 °C) was added to each tube to a final concentration of 1 mM. Resuspended S100A8 and S100A9 were combined in an ice-cold stainless steel beaker, and the mixture was lysed by sonication on ice (30 sec on, 10 sec off for 2.5 min at 40% amplitude). The crude lysate was clarified by centrifugation (14,000 rpm, 20 min, 4 °C), and the supernatant was decanted. S100A8 and S100A9 were retained in the insoluble pellet. The resulting pellet was therefore resuspended in 60 mL of lysis buffer A, and a 600-µL aliquot of PMSF was added. The sonication and centrifugation steps were repeated, which yielded an off-white pellet. This pellet was resuspended in 60 mL of lysis buffer A, and sonicated for a third time. After centrifugation, the resulting cell pellet was suspended in 120 mL of lysis buffer B (50 mM Tris, 100 mM NaCl, 10 mM BME, 4 M Gdn-HCl, pH 8.0) by either gentle stirring at 4 °C or by using a tissue homogenizer (Kontes). Once the pellet containing S100A8 and S100A9 was completely solubilized, the clear solution was sonicated (30 sec on, 10 sec off, 5 min at 40%
amplitude) on ice and centrifuged (14,000 rpm, 20 min, 4 °C). The resulting supernatant was transferred to a Spectropor3 3500 MWCO dialysis bag and dialyzed against 20 mM HEPES pH 8.0 buffer (3 x 4 L, 12-24 h per dialysis, 4 °C). A white precipitate formed during this step, and SDS-PAGE indicated that it mostly contained E. coli proteins (Figure 2.3.1). This mixture was centrifuged (3,750 rpm, 20 min, 4 °C) and passed through a 0.45-μm filter to remove particulates.

Chromatographic purification of CP was performed by using an ÄKTA Purifier FPLC system (GE Lifesciences) housed in a cold room at 4 °C. The A8/A9-containing dialysate was loaded into a 150-mL Superloop (GE Lifesciences). Crude heterodimeric CP was first purified by anion exchange chromatography by using a MonoQ 10/100 GL column and a gradients of 0-30% B over 15 column volumes (eluent A: 20 mM HEPES, 10 mM β-mercaptoethanol (BME), pH 8.0; eluent B: 20 mM HEPES, 10 mM BME, 1 M NaCl, pH 8.0). This step allowed for separation of CP from the S100A9 homodimer. Fractions containing CP (as determined by SDS-PAGE, 15% Tris-HCl gel) were pooled, concentrated to ca. 10 mL, and purified by gel filtration chromatography (20 mM HEPES, 100 mM NaCl, 10 mM BME, pH 8.0) using a HiLoad 26/600 S75 pg column (GE Lifesciences). Fractions containing pure CP were pooled, transferred to a SpectraPor3 3500 MWCO dialysis bag, and dialyzed against 1 L of metal-free 20 mM HEPES, 10 mM BME, 100 mM NaCl, pH 8.0 containing ca. 10 g of Chelex resin (Biorad) at 4 °C for 12-24 h. The dialyzed protein was concentrated in an Amicon 10-kDa MWCO spin filter (3,750 rpm, 4 °C), aliquoted into sterile microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at -80 °C. The yield for wild-type CP was routinely ca. 80 mg / 2 L of E. coli culture (i.e. a 1-L pellet each of S100A8 and S100A9 were employed).

The overexpression, reconstitution, and purification of all heterodimeric CP mutants were conducted as described above with the exception that BME was omitted from all of the buffers. The yields for the mutant proteins ranged from ca. 10 mg/2 L for ΔΔ to ca. 45 mg/2L
for CP-Ser and the metal-binding site mutants. The CP, CP-Ser, ΔHis$_3$Asp, ΔHis$_4$, and ΔΔ proteins were all overexpressed and purified multiple times, and samples from multiple preps were used throughout this work with high reproducibility. Tables 2.2.4 – 2.2.6 include protein nomenclature, molecular weight, and extinction coefficient data for wild-type CP and the mutant family members. For mutant such as ΔΔ that were routinely obtained in lower yields, the purification scale was routinely increased to an 8-L scale. The same buffer volumes described above were used; however, an extra sonication step prior to suspension in lysis buffer B may be included.

The wild-type and mutant proteins were routinely concentrated to >500 μM for storage purposes, aliquoted in 10- to 50-μL portions, and freeze-thawed only once. Prior to an experiment, the protein as thawed on ice and, if necessary, buffer exchanged into the desired buffer.
<table>
<thead>
<tr>
<th>Protein Name</th>
<th>S100A8</th>
<th>Mutations</th>
</tr>
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<tr>
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Table 2.2.5. Molecular Weights and Extinction Coefficients for CP and Mutant Proteins.

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$^a$ Molecular weights were calculated by using the ProtParam tool available on the ExPASy server (http://web.expasy.org/protparam).
$^b$ Extinction coefficients (280 nm) were calculated by using the ProtParam tool available on the ExPASy server (http://web.expasy.org/protparam).
### Table 2.2.6. Molecular Weights and Extinction Coefficients for CP Subunits.

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<sup>a</sup> Molecular weights were calculated by using the ProtParam tool available on the ExPASy server (http://web.expasy.org/protparam).

<sup>b</sup> Extinction coefficients (280 nm) were also calculated by using the ProtParam tool.

### 2.2.5 Protein Mass Spectrometry

An Agilent LC-MS system housing an Agilent 1260 series LC system and an Agilent 6230 TOF system outfitted with an Agilent Jetstream ESI source was employed for high-resolution electrospray ionization mass spectrometry (ESI-MS). An Agilent Poroshell 300SB-
C18 column (5-μm pore size) and a denaturing protocol were utilized for all LC-MS analyses. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. Protein samples (50 μM) were prepared in 20 mM HEPES, 100 mM NaCl, pH 8.0 or Milli-Q water and a 2.5- to 5-μL aliquot was injected onto the Poroshell column for each analysis. The S100A8 and S100A9 subunits were eluted by using a gradient of 0-65% B over 30 min with a flow rate of 0.2 mL/min. The resulting mass spectra were deconvoluted using the maximum entropy algorithm in MassHunter BioConfirm (Agilent).

2.2.6 Analytical Size Exclusion Chromatography

An ÄKTA Purifier (GE Lifesciences) outfitted with a 500-μL sample loop was used for all analytical size exclusion chromatography (SEC). A Superdex 75 10/300 GL column (GE Lifesciences) was calibrated with a 100-μL mixture of aprotinin (6.5 kDa, 3 mg/mL), ribonuclease A (13.7 kDa, 3 mg/mL), carbonic anhydrase (29 kDa, 3 mg/mL), ovalbumin (44 kDa, 4 mg/mL) and conalbumin (75 kDa, 3 mg/mL) obtained from GE Lifesciences. The mixture was buffered at pH 8.0 (20 mM HEPES, 150 mM NaCl). The carbonic anhydrase standard partially precipitated when added to the calibration mixture. The precipitate was pelleted by brief centrifugation, and the supernatant utilized for the column calibration. The dead volume of the column was determined with Blue Dextran 2000 (1 mg/mL) (GE Lifesciences). The partition coefficient, $K_{av}$, for each species was determined by the relationship:

$$K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)}$$

where $V_0$ is the dead volume, $V_e$ is the elution volume of the analyte, and $V_t$ is the bed volume. $K_{av}$ was plotted against the log of the molecular mass of each standard to obtain a linear calibration curve.

In a typical SEC experiment, a 200- to 250-μL sample of 100 or 200 μM CP was loaded into the sample loop. The column was pre-equilibrated with at least one column
volume of running buffer (varied composition), the sample loop was subsequently emptied with 0.5 mL of running buffer, and the sample was eluted over one column volume at a flow rate of 0.5 mL/min. Analytical SEC experiments were conducted at either 4 °C or at room temperature as specified. Theoretical molecular weights were determined from Kav using the linear relationship provided by the calibration curve.

2.2.7 Circular Dichroism Spectroscopy

An Aviv Model 202 circular dichroism (CD) spectrometer thermostatted at 25 °C was utilized to collect CD spectra. A 1-mm path-length quartz CD cell (Hellma) was employed for all CD measurements. Protein solutions (10 μM, 250 – 300 μL) were prepared by diluting an aliquot of a concentrated stock solution into 1 mM Tris-HCl, 500 mM EDTA, pH 8.5. To determine the effect of Ca(II) on the protein fold, an aliquot of 100 mM Ca(II) was added to each sample to provide a final Ca(II) concentration of 1.5 – 2.5 mM and each CD spectrum was recorded ca. 3 min after Ca(II) addition. The CD spectra were collected from 260 – 195 nm at 1 nm intervals (3 sec averaging time, three independent scans per wavelength), and the data obtained from the three scans were averaged and plotted.

2.2.8 Electron Paramagnetic Resonance Spectroscopy

Low-temperature EPR spectra (X-band, 9 GHz) were recorded on a Bruker EMX spectrometer equipped with an ER 4199HS cavity and an Oxford Instruments ESR900 continuous flow liquid helium cryostat. Low-temperature EPR samples were housed in 4-mm (outer diameter) quartz EPR tubes and frozen in liquid nitrogen prior to analysis. The temperature was monitored with either a Cernox sensor or a thermocouple. A copper-EDTA spin standard was used to account for all relevant intensity factors. All samples were measured under non-saturating and saturating conditions. All cobalt spectra were recorded at 9.38 GHz and analyzed with SpinCount, which is available from Prof. Michael Hendrich at Carnegie Mellon University.
2.2.9 Zinc Stoichiometry Determination

In one set of experiments, a 2-mL solution containing ca. 10 μM of CP or mutant and 25 μM Zincon (75 mM HEPES, 100 mM NaCl, pH 7.5) was titrated with Zn(II). The absorbance change at 621 nm was recorded and plotted against the total concentration of Zn(II). In a second set of experiments, an approximately equimolar mixture of CP and MF2 (ca. 10 μM each) was prepared and titrated with Zn(II) (2 mL volume; 75 mM HEPES, 100 mM NaCl, pH 7.5). After each Zn(II) addition, the resulting mixture was incubated at room temperature for 15 min, and the optical absorption spectrum was recorded. The absorbance increase at 325 nm and decrease at 366 nm were recorded and plotted against total Zn(II). The Zn(II) stock solutions were prepared by volumetric dilution of a 100 mM stock solution prepared from ZnCl₂ and water, and the resulting concentrations were verified by using 4-(2-pyridylazo)-resorcinol (PAR, ε ≈ 80,000 M⁻¹ cm⁻¹ for the 1:2 complex at pH 7.3) obtained from Acros Organics. To determine whether Ca(II) influences the Zn:CP stoichiometry, the Zincon experiment was also performed in the presence of 200 pM Ca(II). Zincon has reported Zn(II) Kₐ values of 12.6 and 5.8 μM,²³²⁴ Reported Zn(II) Kₐ values for MF-2 are 20 and 36 nM.²⁵²⁶

2.2.10 Zinc Affinity Determination with FZ3

Solutions containing ca. 2 μM FZ3 and ca. 10 μM CP were prepared at pH 7.5 (75 mM HEPES, 100 mM NaCl), and the emission spectrum was recorded from 500 to 650 nm (λₑₓ = 493 nm). An aliquot of Zn(II) from a freshly prepared 2 mM solution was added, and the solution was mixed in the cuvette and the resulting emission spectrum was recorded. The emission was integrated from 500 to 650 nm by using the KaleidaGraph software package.
2.2.11 Zinc Affinity Determination with ZP4

Competition titrations with CP and ZP4 were employed to determine the dissociation constants of CP for Zn(II). In a typical experiment, an aliquot from a ca. 1 mM DMSO stock solution of ZP4 was diluted to a final concentration of ca. 2 μM in a total volume of 2 mL (75 mM HEPES, 100 mM NaCl, pH 7.5). After the optical absorption ($\varepsilon_{506} = 61,000 \text{ M}^{-1} \text{ cm}^{-1}$)\textsuperscript{27} and emission spectra of this solution were recorded, and aliquot of CP was added to provide a final concentration of ca. 5 or 10 μM as indicated, and the optical absorption and emission spectra were recorded again. The absorption and fluorescence spectra of ZP4 were unperturbed by the addition of CP. This CP/ZP4 mixture was titrated with Zn(II) using a freshly prepared working solution from a 100 mM stock of ZnCl\textsubscript{2} in water that was quantified by PAR. After each Zn(II) addition, the solution was mixed gently and incubated in the dark for a minimum of 10 min. The emission spectrum was recorded from 505 – 650 nm ($\lambda_{\text{em}} = 495 \text{ nm}$), and the resulting spectra were integrated over this range. The integrated emission versus the concentration of Zn(II) added was plotted, and the resulting titration curve was fit to a two-site model using the DynaFit software and a custom script. The dye response factors required by DynaFit were determined for free ZP4 in the presence of 100 μM EDTA and for ZP4 in the presence of 100 μM Zn(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). For CP/ZP4 competition experiments in the presence of Ca(II), the same procedures were employed except that 100 – 450 μM Ca(II) was added to buffer. The reported Zn(II) $K_d$ values were obtained by averaging the $K_d$ values obtained from fitting three independent titrations. The errors are the standard deviations from the mean.

2.2.12 Calcium-Dependent Zn(II) Sequestration by CP

A solution of CP/ZP4 (75 mM HEPES, 100 mM NaCl, pH 7.5) was first titrated with Zn(II) to afford maximum ZP4 turn-on, and an aliquot of Ca(II) from a 100 mM stock solution was added to provide a final Ca(II) concentration of 200 μM. The resulting changes in ZP4
emission were monitored over the course of 300 min. Once the emission intensity returned to the baseline (apo ZP4) value, the solution was titrated with Zn(II) until maximum emission from the Zn:ZP4 complex was restored. The maximal ZP4 emissions achieved before and after Ca(II) addition were in excellent agreement, confirming the integrity of the ZP4 sensor over the course of the experiment.

To probe Zn(II) dissociation from CP (ωC2), a solution containing ca. 6 μM CP, ca. 3 μM Zn(II), and 200 μM Ca(II) was incubated at room temperature for 30 min, and an aliquot of ZP4 was added to provide a final concentration of 2 μM (75 mM HEPES, 100 mM NaCl, pH 7.5). The ZP4 emission was monitored over 300 min. Controls without CP and without Zn(II) were conducted in parallel. With the exception of the no CP control, which exhibited full ZP4 turn-on, addition of 50 μM Zn(II) to the samples after the incubation period resulting in full ZP4 turn on, verifying the integrity of the ZP4 over the course of the experiment.

2.2.13 Antimicrobial Activity Assays

All bacterial strains were purchased from ATCC. To first confirm that recombinant wild-type CP exhibited antibacterial activity, the ability of CP to inhibit the growth of S. aureus ATCC 25923 was monitored by using a modified literature protocol.16 S. aureus ATCC 25923 was grown overnight with shaking (37 °C, 16 h) in 6 mL of TSB without dextrose. The overnight culture was diluted 1:100 into 6 mL of fresh TSB without dextrose and grown for ca. 2 h at 37 °C until the OD600 reached 0.6. The culture was diluted 1:500 in two steps (1:100 then 1:5) into antimicrobial assay media (62:38 ratio of 20 mM Tris-HCl, 100 mM NaCl, 5 mM BME, ±3 mM Ca(II), pH 7.5, and TSB without dextrose) and used immediately. Protein aliquots were thawed on ice, diluted 10-fold with AMA buffer (20 mM Tris-HCl, 100 mM NaCl, 5 mM BME, pH 7.5), and buffer exchanged three time using a 10-kDa MWCO 0.5-mL Amicon microcentrifuge tube (Millipore). The resulting protein concentrations were determined by $A_{280}$, and the aliquots diluted with AMA buffer to provide
10x protein stocks (5000 μg/mL to 312.5 μg/mL). The antibacterial activity assays were performed in polystyrene 96-well plates (Corning). Each well contained a 90-μL aliquot of the diluted S. aureus culture and a 10-μL aliquot of the 10x protein stock solutions or a no-protein control (AMA buffer). Each condition was prepared in triplicate. Each plate was wrapped with parafilm and incubated at 37 °C with shaking at 150 rpm in a tabletop incubator shaker housing a beaker of water. The OD<sub>600</sub> values were measured at varying timepoints (0-24 h) by using a plate reader. The assay was also performed with CP-Ser. For assays conducted with Ca(II)-supplemented media, the final concentration of Ca(II) resulting from the supplement was 2 mM.

To investigate the antibacterial activity of CP and CP-Ser against E. coli ATCC 25922 and Enterobacter aerogenes ATCC 13048, growth assays were conducted as described above with the following modifications: (i) the TSB contained dextrose, (ii) the assays were conducted at 30 °C, (iii) the OD<sub>600</sub> values were recorded after a 24 h incubation, and (iv) no beaker of water was housed in the incubator shaker. These assays were also conducted the absence and presence of a 2 mM Ca(II) supplement.

To ascertain the effect of supplementing AMA assay media with CP, CP-Ser, ΔHis<sub>3</sub>Asp, ΔHis<sub>4</sub>, or ΔΔ on the growth of a variety of bacterial species, a series of assays was conducted as described above for S. aureus ATCC 25923 except that (i) the assays were conducted at 30 °C, (ii) a single protein concentration of 500 μg/mL was employed, and (iii) the OD<sub>600</sub> was monitored at t = 8 h and t = 20 h. S. aureus ATCC 25923, E. coli ATCC 25922, and E. aerogenes ATCC 13048 were investigated.

For all antibacterial activity assays, each experiment was repeated a minimum of three times and with at least two independent single-colony cell stocks of each bacterial species.
2.3 Results and Discussion

2.3.1 Design, Preparation, and Characterization of a Calprotectin Mutant Family

In order to study the metal-binding properties of CP, a mutant family was designed, overexpressed, and purified. To facilitate metal-binding studies in the absence of an external reducing agent such as dithiothreitol (DTT) or β-mercaptoethanol (BME), Cys42 of S100A8 and Cys3 of S100A9 were mutated to serine to afford CP-Ser. CP-Ser was purified using the denaturing protocol as described above, and a representative purification gel is shown in Figure 2.3.1.

Figure 2.3.1. SDS-PAGE (15% Tris-HCl gel) of samples from a representative preparation of CP-Ser. S100A8 is 10.8 kDa and S100A9 is 13.2 kDa. Lane 1: P7708S pre-stained gel ladder (New England Biolabs). Lanes 2 and 3: overexpression of S100A8(C42S) showing pre- and post-induction samples. Lanes 4 and 5: overexpression of S100A9(C3S) showing pre- and post-induction samples. Lane 6: the soluble fraction after combining the A8 and A9 cell pellets and lysis. Lane 7: the insoluble fraction after cell lysis. Lane 8: Soluble CP-Ser after refolding. Lanes 9-11: Fractions containing CP-Ser obtained from anion-exchange chromatography. Lanes 12-15: fractions containing pure CP-Ser from gel-filtration chromatography.
Amino acids comprising the putative metal-binding site (Figure 2.2.1) were systematically mutated to non-coordinating alanine. The pET41a-S100A8(C42S) and pET41a-S100A9(C3S) plasmids were used as the templates for site-directed mutagenesis (Tables 2.2.1-2.2.3). Metal-binding site mutants with (i) single point mutations, (ii) two mutations in the His$_3$Asp or His$_4$ site, and (iii) deleted sites were purified as described above. Each protein was characterized by SDS-PAGE (Figure 2.3.2), ESI mass spectrometry (Table 2.3.1), circular dichroism spectroscopy (Figure 2.3.3 and Appendix 2), and analytical size exclusion chromatography (Figure 2.3.4, Table 2.3.2 and Appendix 2). SDS-PAGE revealed that all proteins were obtained in high purity (Figure 2.3.2), and ESI-MS confirmed that each subunit had the correct expected mass (Table 2.3.1). CD spectroscopy of CP-Ser revealed minima at 208 nm and 222 nm (Figure 2.3.3 and Appendix 2), which is consistent with the expected $\alpha$-helical secondary structure of CP and is in agreement with published spectra. The CD spectrum of CP-Ser is largely unperturbed by the addition of excess Ca(II) (Figure 2.3.3 and Appendix 2). The mutations at the metal-binding sites did not perturb the secondary structure of CP-Ser. Analytical SEC established that each protein was isolated as the $\alpha\beta$ dimer, and that the addition of excess Ca(II) induced the formation of the expected $\alpha_2\beta_2$ tetramer (Figure 2.3.4 and Appendix 2). The elution volumes of the major species, as well as the calculated molecular weights, are given in Table 2.3.2. In the absence of Ca(II), CP-Ser eluted at 10.8 mL, corresponded to 36 kDa and assigned to the $\alpha\beta$ dimer. Addition of 2 mM Ca(II) to the running buffer resulted in disappearance of the peak at 10.8 mL and appearance of a new peak at 10.2 mL, corresponded to a 48-kDa species and assigned as the $\alpha_2\beta_2$ tetramer. In some cases, evidence for higher-order oligomers were also observed in the form of small shoulders at lower elution volumes.
Figure 2.3.2. SDS-PAGE (15% Tris-HCl gel) of purified proteins employed in this study. S100A8 is 10.8 kDa and S100A9 is 13.2 kDa. Top panel A: Single and double mutants of the His$_3$Asp and His$_4$ sites. (A) P7708S pre-stained gel ladder; (B) CP-Ser(H20A); (C) CP-Ser(D30A); (D) CP-Ser(H83A); (E) CP-Ser(H87A); (F) CP-Ser(D30A)(H83A); (G) CP-Ser(H17A); (H) CP-Ser(H27A); (I) CP-Ser(H91A); (J) CP-Ser(H95A); (K) CP-Ser(H27A)(H91A). Bottom panel B: Wild-type CP and metal-binding site mutants. (A) P7710S pre-stained gel ladder; (B) CP; (C) CP-Ser; (D) CP-Ser ΔHis$_3$Asp; (E) CP-Ser ΔHis$_4$; (F) CP-Ser ΔΔ; (G) P7710S.
Table 2.3.1. Summary of Results from Mass Spectrometry of CP and Mutants.

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* A denaturing protocol on an Agilent Poroshell 300SB-C18 column over a 0 – 65 % gradient of acetonitrile in 0.1 % formic acid was employed for LC-MS. * Molecular weights were calculated by using the ProtParam tool available on the ExPASy server (http://web.expasy.org/protparam). * Masses were calculated with the Agilent MassHunter BioConfirm software package. * The N-terminal methionine of S100A9 is sometimes cleaved during overexpression in E. coli. Masses are shown for the full-length and shortened forms of S100A9. * Mass not found following deconvolution of the raw data.
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* Protein (100 μM) was eluted over a S75 10/300 GL SEC column (GE Healthsciences) with 75 mM HEPES, 100 mM NaCl, pH 7.5 and a 0.5 mL/min flow rate. Molecular weights were calculated with a calibration curve spanning a range of 6.5 – 75 kDa.
**Figure 2.3.3.** CD spectra of CP-Ser in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA, T = 25 °C).

**Figure 2.3.4.** Analytical size exclusion chromatography of 100 μM CP-Ser in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5) (A) Full chromatograms. (B) Expansion. The red star indicates Ca(II)-dependent peaks attributed to higher-order oligomers. Absorption was monitored at 280 nm and room temperature. Elution volumes are listed in Table 2.3.2.

Titrations of ca. 100 μM CP or CP-Ser with 0-8 equivalents of Ca(II) were monitored by analytical SEC and resulted in a stepwise decrease in the peak 10.8 mL with concomitant growth of the 10.2 mL peak (Figure 2.3.5). Complete conversion of αβ CP to the α₂β₂ tetramer was not observed indicating that either (i) 8 equivalents of Ca(II) are insufficient to occupy the available EF-hands, or (ii) Ca(II) is lost from the EF-hands during the elution. Both of these possibilities may be explained by the EF-hands having a relatively low affinity for Ca(II).
for Ca(II). Indeed, the non-canonical N-terminal EF-hands of S100A8 and S100A9 contribute mostly neutral ligands to ion coordination and are expected to have relatively low affinities for Ca(II).\textsuperscript{19}

\textbf{Figure 2.3.5.} Calcium-binding titrations monitored by SEC at pH 8.0 (20 mM HEPES, 100 mM NaCl) of CP (A, 135 μM) and CP-Ser (B, 122 μM). CP was eluted following the addition of 0, 1, 2, 4, and 8 equivalents of Ca(II). The arrows indication conversion of the αβ dimer to the α₂β₂ tetramer with the addition of Ca(II). The absorbance was monitored at 280 nm and T = 4 °C.

We examined the influence of Ca(II) on the stability of the CP-Ser secondary structure by performing thermal denaturation and monitoring the CD signal at 222 nm. Apo CP-Ser (αβ) gave a melting temperature (T\textsubscript{m}) of 59 °C at pH 8.5 (1.0 mM Tris, 0.5 mM EDTA). In the presence of 2 mM Ca(II), the T\textsubscript{m} of CP-Ser (α₂β₂) shifted by 20 °C to 79 °C (Figure 2.3.6). Taken together with the analytical SEC observations, these data indicate that Ca(II)-binding and tetramer formation stabilize the α-helical structure of CP to thermal denaturation. These data are in general agreement with differential scanning calorimetry of CP.\textsuperscript{28} It is unclear if this observation is due to formation of the tetramer, or if Ca(II)-coordination by the EF-hands is sufficient to stabilize the secondary structure of CP.
2.3.2 Calprotectin Binds Co(II) at the Interfacial His₃Asp and His₄ Motifs

High-spin Co(II) is a 3d⁷ metal that is a useful spectroscopic probe for closed shell Zn(II) (3d¹⁰). The Co(II) ligand field transitions can be readily observed by optical absorption spectroscopy and readily correlated to the coordination geometry and ligand environment. Addition of 0-5 equivalents of Co(II) to CP-Ser (400 µM, αβ) at pH 7.0 (75 mM HEPES, 100 mM NaCl) resulted in the formation of a pink solution with a d-d transition centered at 556 nm (ε = 480 M⁻¹ cm⁻¹) (Figure 2.3.7 panel A). A similar feature with a lower extinction coefficient (λ_max = 556 nm, ε = 280 M⁻¹ cm⁻¹) was observed during titration of CP-Ser-ΔHis₄ (400 µM, αβ), and a weak absorption feature centered at 499 nm (ε = 38 M⁻¹ cm⁻¹) formed following addition of Co(II) to a sample of CP-Ser-ΔHis₃Asp (400 µM, αβ) (Figure 2.3.7 panels B and C; Figure 2.3.8). The addition of 2 mM Ca(II) had negligible effect on the absorption spectrum of cobalt-bound CP-Ser (100 µM, αβ₂, Figure 2.3.9); however, titration of Co(II) into a solution of Ca(II)-bound CP caused precipitation at higher concentrations of CP.

![Figure 2.3.6.](image-url) Thermal denaturation of 10 µM CP-Ser at pH 8.5 (1 mM Tris, 0.5 mM EDTA) in the absence (black circles) and presence (red squares) of 2 mM Ca(II). Circular dichroism at 222 nm was monitored. The denaturation curves afford T_m values of 59 °C and 79 °C in the absence and presence of Ca(II), respectively.
Figure 2.3.7. Cobalt binding to CP monitored by optical absorption spectroscopy. CP (400 μM) was titrated with 0-5 equivalents of Co(II) at pH 7.0 (75 mM HEPES, 100 mM NaCl) and 25 °C. (A) Titration of CP-Ser with Co(II). A d-d transition centered at 556 nm ($\varepsilon = 480$ M$^{-1}$ cm$^{-1}$) is observed. (B) Titration of CP-Ser-ΔHis$_4$ with Co(II). A d-d transition centered at 556 nm ($\varepsilon = 280$ M$^{-1}$ cm$^{-1}$) is observed. (C) Titration of CP-Ser-ΔHis$_3$Asp with Co(II). A d-d transition centered at 499 nm ($\varepsilon = 38$ M$^{-1}$ cm$^{-1}$) is observed. An expansion is shown in Figure 2.3.8.

Figure 2.3.8. Titration of 400 μM CP-Ser-ΔHis$_3$Asp with 0-5 equivalents of Co(II) monitored by optical absorption spectroscopy at pH 7.0 (75 mM HEPES, 100 mM NaCl). The inset contains an expanded y-axis of the plot.
Figure 2.3.9. Titration of 100 μM CP-Ser with 0-3 equivalents of Co(II) in the presence of 2 mM Ca(II) monitored by optical absorption spectroscopy at pH 7.0 (75 mM HEPES, 100 mM NaCl) and 25 °C. Precipitation occurred at high equivalents of Co(II).

The $\lambda_{\text{max}}$ values and extinction coefficients for the CP mutants indicate that both sites 1 and 2 coordinate Co(II) and do so with different geometries. In a tetrahedral ligand environment with N- and O-donor ligands, Co(II) typically exhibits $\varepsilon \geq 300$ M$^{-1}$ cm$^{-1}$. Five- and six-coordinate Co(II) typically display less intense transitions of $250$ M$^{-1}$ cm$^{-1} > \varepsilon > 50$ M$^{-1}$ cm$^{-1}$ and $\varepsilon < 50$ M$^{-1}$ cm$^{-1}$. Because of different ligand field stabilization energies, the $\lambda_{\text{max}}$ values also vary with coordination number from $650 \pm 50$ nm (tetrahedral, four-coordinate) to $525 \pm 50$ nm (six-coordination, octahedral). The optical absorption spectra for the CP mutants indicate that Co(II) is coordinated in a four- or five-coordinate manner at the His$_3$Asp site, suggesting that the Asp residue may be mono- or bidentate. The His$_4$ site likely provides six-coordinate geometry, indicating that as-yet unidentified water molecules, buffer components, or protein residues may contribute to the coordination sphere at site 2.

Cobalt complexation by CP was further evaluated by low-temperature EPR spectroscopy, and this technique provided unambiguous evidence for two distinct Co(II)-
binding sites (Figures 2.3.10 and 2.3.11). High-spin Co(II) has a spin of \( S = \frac{3}{2} \), and the \(^{59}\text{Co}\) nucleus has a magnetic moment of \( I = \frac{7}{2} \). Addition of 0.8 equivalents of Co(II) to CP-Ser (1.1 mM) at pH 7.0 (75 mM HEPES, 100 mM NaCl) gave rise to a broad EPR signal at 10.6 K that is typical of high-spin Co(II) with no observable nuclear hyperfine structure. The signal contained absorption-like and derivative features at \( g_{\text{eff}} = 6.04 \) and 4.1, respectively. Unique EPR signals for Co(II) bound to CP-Ser-\( \Delta \text{His}_3\text{Asp} \) and CP-Ser-\( \Delta \text{His}_4 \) were observed and support different coordination motifs (Figure 2.3.10). The CP-Ser-\( \Delta \text{His}_3\text{Asp} \) Co(II) signal was dominated by a sharp derivative feature at \( g_{\text{eff}} = 4.0 \). The signal of Co(II) coordinated by CP-Ser-\( \Delta \text{His}_4 \) was markedly less intense and contained both absorption-like and derivative features at \( g_{\text{eff}} = 6.06 \) and 4.14, respectively. Addition of Co(II) to CP-Ser-\( \Delta \Delta \) resulted in a signal resembling the Co(II) in buffer standard. Linear combination of the Co(II) / CP-Ser-\( \Delta \text{His}_3\text{Asp} \) and Co(II) / CP-Ser-\( \Delta \text{His}_4 \) closely reproduced the CP-Ser signal and provided \( \Delta \text{His}_3\text{Asp} / \Delta \text{His}_4 \) ratios of 2:1 to 1:2, depending on the sample. Inclusion of the \( \Delta \Delta \) signal had negligible impact on the reproduction of the CP-Ser signal. Power saturation of the CP-Ser signal at 10.6 K resulted in different saturation behavior in different regions of the spectrum, supporting the presence of multiple Co(II) coordination motifs.\(^{30,31}\) Moreover, power saturation of the CP-Ser-\( \Delta \text{His}_3\text{Asp} \) and CP-Ser-\( \Delta \text{His}_4 \) samples revealed unique saturation behaviors that correlate with the changes in signal intensity in the CP-Ser sample (Figure 2.3.11). These studies confirm that both sites 1 and 2 coordinate Co(II), and both sites are occupied in the presence of substoichiometric Co(II).

The CD spectrum of CP-Ser (10 \( \mu \text{M} \)) was unperturbed by the addition of 10 equivalents of Co(II) in the presence and absence of Ca(II), revealing that Co(II) binding has negligible impact on the overall secondary structure of CP (Figure 2.3.12).
Figure 2.3.10. Low-temperature EPR spectroscopic signals of Co(II)-bound CP. (A) EPR spectra of 1.1 mM CP-Ser and the indicated metal-binding site mutants in the presence of 0.8 equivalents Co(II) at pH 7.0 (20 mM HEPES, 100 mM NaCl). The colors of the CP-Ser and ΔHis₄ spectra indicate the color of the samples following Co(II) addition. The samples of ΔHis₃Asp, ΔΔ, as well as the buffer reference were colorless. Instrument conditions: temperature, 10.6 K; microwaves, 2 mW at 9.4 GHz; modulation amplitude, 8.0 G. (B) The black trace is a linear combination of the ΔHis₄ and ΔHis₃Asp signals shown in panel A. Combination of ½ ΔHis₄ and ½ ΔHis₃Asp closely reproduces the CP-Ser signal shown in purple. (C) Power saturation experiments. EPR spectra of the CP-Ser sample from panel A were recorded at 10.6 K with powers of 0.6, 2, 6, and 20 mW. The grey scale indicates increasing power from dark to light grey.

Figure 2.3.11. EPR power saturation experiments. (A) CP-Ser-ΔHis₃Asp. (B) CP-Ser-ΔHis₄. EPR spectra were recorded at 10.6 K with powers of 0.6 (dark grey), 2, 6, and 20 (light grey) mW. The samples contained 1.1 mM CP and 0.8 equivalents of Co(II) at pH 7.0 (20 mM HEPES, 100 mM NaCl). Instrument conditions: temperature, 10.6 K; microwaves, 9.4 GHz; modulation amplitude, 8.0 G.
2.3.3 Calprotectin Coordinates Zinc(II) at the Interfacial His₃Asp and His₄ Sites

We hypothesized that site 1 and site 2 of calprotectin may also contribute to Zn(II) coordination. Addition of Zn(II) to a 1:1 mixture of CP-Ser (100 µM) and Co(II) at pH 7.0 (75 mM HEPES, 100 mM NaCl) resulted in a gradual decrease in the electronic transition centered at 556 nm over the course of 1 h (Figure 2.3.13). This observation indicates that Zn(II) displaces Co(II) from at least the His₃Asp Co(II) binding site(s) of CP-Ser, as expected based on the Irving-Williams series. Titration of CP-Ser (400 µM, 75 mM HEPES, 100 mM NaCl, pH 7.0) with a 1:1 mixture of Co(II):Zn(II) resulted in negligible change in the absorption at 556 nm, revealing that Zn(II) prevented Co(II) coordination by CP-Ser at the His₃Asp site (Figure 2.3.14). The absorptivity of Co(II) bound to the His₄ site is weak, and these sets of experiments do not provide conclusive evidence that Zn(II) coordinates to site 2. Taken together, these results suggest that Zn(II) and Co(II) share the same binding site(s) in CP-Ser.
Figure 2.3.13. Optical absorption spectra revealing displacement of Co(II) from 100 μM CP-Ser by Zn(II) addition at pH 7.0 (75 mM HEPES, 100 mM NaCl) and T = 25 °C. Red line: optical absorption spectrum of CP-Ser in the presence of 1 equivalent of Co(II). Black lines: Optical absorption spectra immediately after the addition of 1 equivalent of Zn(II) at t = 15, 30, 45, 60, and 90 min post addition of Zn(II). Blue lines: Optical absorption spectra immediately (top) and 60 (bottom) after addition of a second equivalent of Zn(II).

Figure 2.3.14. Titration of 400 μM CP-Ser with Co(II) or a 1:1 mixture of Co(II):Zn(II) at pH 7 (75 mM HEPES, 100 mM NaCl) monitored by optical absorption spectroscopy (T = 25 °C). The Co(II) titration data correspond to the optical absorption spectra presented in Figure 2.3.7. Each optical absorption measurement was made ca. 1 min after addition of the metal solution to the cuvette.
In order to ascertain the Zn / CP stoichiometries, we employed a strategy in which we titrated Zn(II) into a mixture of CP (or mutant protein) and a colorimetric Zn(II) indicator. Zincon is a relatively low-affinity Zn(II) chelator ($K_d \approx 10 \mu M$), and titration of a 2:1 mixture of Zincon:CP afforded a colorimetric response only after 2 equivalents of Zn(II) / CP-Ser were added to the solution. This observation indicates that CP-Ser coordinates 2 equivalents of Zn(II) with higher affinity than Zincon ($K_d \leq 10^{-6} M$). Moreover, titration of 2:1 mixtures of Zincon / CP-Ser-$\Delta$His$_3$Asp or Zincon / CP-Ser-$\Delta$His$_4$ afforded a colorimetric response after 1 equivalent of Zn(II) / CP was added, confirming that site 1 and site 2 coordinate Zn(II). Titration of Zincon with Zn(II) in the presence of CP-Ser-$\Delta\Delta$ resulted in a small perturbation of the colorimetric response, indicating that CP-Ser-$\Delta\Delta$ mutant does not coordinate Zn(II) with sub-micromolar affinity. This result is in general agreement with ITC experiments that were previously performed with a $\Delta$Zn/Mn multiple mutant.\textsuperscript{16}

**Figure 2.3.15.** Zinc response of 25 µM Zincon in the presence of ca. 10 µM CP-Ser at pH 7.5 (75 mM HEPES, 100 mM NaCl) at T = 25 °C. The absorption values at 621 nm indicate that Zincon only responds after CP-Ser binds 2 equivalents (red circles) or the CP-Ser-$\Delta$His$_3$Asp (blue squares) and CP-Ser-$\Delta$His$_4$ (green triangles) each coordinate 1 equivalent of Zn(II). Negligible attenuation of the Zincon response is observed in the presence of CP-Ser-$\Delta\Delta$ (black diamonds).

To further probe Zn(II)-binding by CP-Ser, we employed a series of Zn(II) competitions using the turn-on fluorescence Zn(II) indicator FZ3 ($K_{d,Zn(II)} = 9 \text{nM}$).\textsuperscript{33} No change in FZ3 emission was observed following addition of 2 µM Zn(II) to a mixture of 2 µM
FZ3 and 10 μM CP-Ser (Figure 2.3.16 panel A). CP-Ser outcompetes FZ3 for Zn(II), and this observation indicates that CP-Ser coordinates Zn(II) with sub-nanomolar affinity. Zinc addition to FZ3 in the presence of CP-Ser-ΔHis3Asp and CP-Ser-ΔHis4 afforded ca. 17- and 2-fold fluorescence enhancements, respectively (Figure 2.3.16). These experiments suggest an apparent $K_a$ ordering of CP-Ser < ΔHis4 < ΔHis3Asp.

Figure 2.3.16. Fluorescence response of 2 μM FZ3 to 2 μM Zn(II) in the presence of 10 μM CP-Ser at pH 7.5 (75 mM HEPES, 100 mM NaCl) and $T = 25^\circ$C. (A) No fluorescence change is observed following addition of Zn(II) to mixtures of FZ3 and CP-Ser. Inset: Expansion of the y-axis. Dotted line, FZ3 emission in the absence of Zn(II); solid red line, FZ3 emission after addition of Zn(II) in the presence of CP-Ser. (B) Fluorescence enhancement in observed for FZ3 in the presence of CP-Ser-ΔHis4 (ca. 2-fold, green line) and CP-Ser-ΔHis3Asp (ca. 17-fold, blue line). FZ3 in the absence of protein (e.g. no CP, black line) exhibits ca. 40-fold fluorescence turn-on following the addition of 1 equivalent of Zn(II). The maximum emission for FZ3 in the presence of 1 equivalent of Zn(II) was adjusted to an integrated emission value of 100, and the remaining emission spectra were scaled accordingly.

2.3.4 Calprotectin Coordinates Zinc(II) with Picomolar Affinity in a Ca(II)-Dependent Manner

We next employed the Ca(II)-insensitive turn-on fluorescent Zn(II) sensor ZP4 ($K_{d,Zn(II)} = 0.65 \pm 0.1$ nM) to evaluate the Zn(II)-binding properties of heterodimeric CP-Ser. Titration of Zn(II) into a mixture of ZP4 and CP-Ser revealed that CP-Ser competes with ZP4 for Zn(II) (Figure 2.3.17). The response curves of ZP4 in the presence of CP-Ser were fit to
a two-site model using DynaFit to afford the following affinities of CP-Ser for Zn(II): $K_{d,site1} = 133 \pm 58$ pM and $K_{d,site2} = 185 \pm 219$ nM. Guided by the FZ3 competition experiments, the picomolar and nanomolar affinities are assigned to the His$_3$Asp and His$_4$ motifs, respectively. These assignments are also supported by competition titrations performed with the CP-Ser(D30A) single mutant and the CP-Ser(H27A)(H91A) double mutant (Figure 2.3.18). Mutation of (S100A9)Asp30 to non-coordinating alanine markedly reduced the ability of CP-Ser to compete with ZP4 for Zn(II); in contrast, mutation of (S100A8)His27 and (S100A9)His91 had a negligible effect on the response curve of the ZP4 / CP mixture. Based on the FZ3 competition, we contend that the affinity of site 2 for Zn(II) is likely in the low-nanomolar range.

![Figure 2.3.17](image)

**Figure 2.3.17.** Fluorescence response of 2 µM ZP4 to Zn(II) in the presence of 10 µM CP-Ser at pH 7.5 (75 mM HEPES, 100 mM NaCl) and $T = 25 \, ^\circ C$. Each emission spectrum was integrated and the resulting values were normalized to the maximum response and plotted against equivalents of Zn(II) per CP-Ser ($\alpha\beta$). The black squares indicate the response in the absence of Ca(II), and the red circles indicate the titration performed in the presence of 200 µM Ca(II). A representative titration for each set of conditions is shown. The titrations were fit to a two-site binding model. The black and red lines represent the fits. The $K_d$ values are $K_{d,site1} = 133 \pm 58$ pM (-Ca), $\leq 10$ pM (+Ca) and $K_{d,site2} = 185 \pm 219$ nM (-Ca), $\leq 240$ pM (+Ca). Excitation was provided at 495 nm and the emission spectra were integrated from 500 – 650 nm.
Figure 2.3.18. Fluorescence response of 2 μM ZP4 to Zn(II) in the presence of ca. 5 μM CP-Ser (black circles), the CP-Ser(H27A)/(H91A) double mutant (green squares), or the CP-Ser(D30A) single mutant (blue triangles). Each emission spectrum was integrated, and the resulting values were normalized to the maximum response. For all titrations, excitation was provided at 495 nm and the emission spectra were integrated from 500 – 650 nm.

ZP4 is a Ca(II)-insensitive sensor, and this feature facilitated ZP4 / CP competition experiments in the presence of excess Ca(II) where the heterotetramer CP oligomer predominates. When Zn(II) was titrated in to a solution containing 2 μM ZP4, 10 μM CP-Ser, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl), the response of ZP4 was attenuated relative to the curve observed in that absence of Ca(II) (Figure 2.3.17). The fluorescence emission of ZP4 began to increase only after ca. 1.5 equivalents of Zn(II) / CP-Ser had been added to the solution, indicating that CP-Ser has a higher affinity for Zn(II) in the presence of Ca(II). Both the CP-Ser-ΔHis3Asp and CP-Ser-ΔHis4 mutants outcompeted ZP4 for Zn(II) in the presence of Ca(II) (Figure 2.3.19), indicating that the affinity for Zn(II) has increased for both site 1 and site 2. The +Ca(II) response curve of titration with CP-Ser could be fit with Dynafit to a two-site model with $K_{d,site1} \leq 10$ pM and $K_{d,site2} \leq 240$ pM. Because CP-Ser outcompetes ZP4 for Zn(II) in the presence of Ca(II), these values provide an upper limit for the dissociation constants. Addition of Zn(II) to samples of CP-Ser (± Ca)
did not perturb the overall secondary structure of CP-Ser (Figure 2.3.20). This result is in agreement with the CD spectrum of wild-type CP in the presence of Zn(II). \(^{28}\)

**Figure 2.3.19.** Fluorescence response of ca. 2 µM ZP4 to Zn(II) in the presence of CP-Ser ΔHis\(_3\)Asp and ΔHis\(_4\). (A) Response of ZP4 to Zn(II) in the presence of ΔHis\(_3\)Asp. Black circles: 11.2 µM CP-Ser ΔHis\(_3\)Asp, no added calcium. Red squares: 9.9 µM ΔHis\(_3\)Asp and in the presence of 200 µM Ca(II). (B) Response of ZP4 to Zn(II) in the presence of ΔHis\(_4\). Black circles: 7.9 µM ΔHis\(_4\), no added calcium. Red square: 7.6 µM ΔHis\(_4\) and in the presence of 200 µM Ca(II). All titrations were conducted at pH 7.5 (75 mM HEPES, 100 mM NaCl) and at 25 °C. Excitation was provided at 495 nm and the emission spectra were integrated from 500 – 650 nm.

The dissociation constants of CP-Ser for Zn(II) are different from those previously reported from ITC measurements. \(^{16}\) Previous studies performed with wild-type CP in the presence of BME and "stoichiometric" Ca(II) provided dissociation constants for Zn(II) of \(K_{d1} = 1.4\) nM and \(K_{d2} = 5.6\) nM. \(^{16}\) It is unclear if stoichiometric Ca(II) refers to the Ca(II) concentration per CP dimer, CP tetramer, or EF-hand. Moreover, the resulting isotherms indicate stoichiometric binding, which makes it difficult to fit unique thermodynamic solutions. \(^1\) The discrepancies between the two sets of experiments may be explained by the limited sensitivity of ITC, which cannot provide accurate dissociation constants in the picomolar range, or differences in the sample conditions. The ITC experiments were performed with Tris buffer and BME, both of which may complex transition metal ions.
Furthermore, the studies presented here indicate that an excess of Ca(II) is necessary for complete conversion to the α₂β₂ tetramer, and a mixture of oligomeric states may be present in the ITC sample.

**Figure 2.3.20.** CD spectra of 10 μM CP-Ser in the absence and presence of 3 equivalents of Zn(II) at pH 7.5 (1 mM Tris). The black line is the CD spectrum of CP-Ser in the absence of any metal ions. The red line is the spectrum of CP-Ser in the presence of 3 equivalents of Zn(II). The blue line is the CD spectrum of CP-Ser in the presence of Co(II) and 2 mM Ca(II).

### 2.3.5 Antibacterial Activity of CP Is Ca(II)-Dependent and Requires at Least One Metal-Binding Site

The growth inhibitory activity of wild-type CP and CP-Ser against *S. aureus* ATCC 25923 was evaluated to confirm the integrity and activity of the recombinant proteins (Figure 2.3.21 panel C and Figure 2.3.22). Negligible growth of *S. aureus* ATCC 25923 occurred in the presence of 500 μg/mL CP and 2 mM Ca(II) under standard *in vitro* assay conditions. This result is in general agreement with previously published work.¹⁵,¹⁶ In contrast, antibacterial assays conducted in media lacking a 2-mM Ca(II) supplement revealed that CP and CP-Ser do not inhibit *S. aureus* ATCC 25923 growth under these conditions. CP and CP-Ser also inhibited the growth of *E. coli* ATCC 25922 and *E. aerogenes* ATCC 13048,
and an increase in potency was observed in Ca(II)-replete conditions. These observations indicate that (i) the (S100A8)Cys42 and (S100A9)Cys3 residues are not essential for antibacterial activity and (ii) CP and CP-Ser exhibit Ca(II)-dependent growth inhibition against several bacterial species. A prior report indicated that (S100A9)Cys3 was required for growth inhibition against S. aureus strain Newman; however, subsequent reports refute this notion. Taken together with the ZP4 competitions, these assays indicate that the CP heterotetramer, which has at least picomolar affinity for Zn(II), is necessary for growth inhibition of some microorganisms.

Figure 2.3.21. Wild-type CP and CP-Ser exhibit calcium-dependent antibacterial action for both Gram-negative and Gram-positive species. (A) Enterobacter aerogenes ATCC 13048, (B) Escherichia coli ATCC 25922, and (C) Staphylococcus aureus ATCC 25923. The black traces are cultures treated with CP (circles) or CP-Ser (squares) in the absence of a 2 mM Ca(II) supplement. The red traces are for cultures treated with CP (circles) or CP-Ser (squares) in the presence of a 2 mM Ca(II) supplement. The OD values were recorded at t = 24 h (mean ± SEM for three independent replicates).
Figure 2.3.22. Growth inhibitory activity of CP and CP-Ser against *S. aureus* ATCC 25923 in the absence and presence of a 2 mM Ca(II) supplement (media here). (A) Wild-type CP in the absence of added Ca(II). (B) Wild-type CP in the presence of 2 mM Ca(II). (C) CP-Ser in the absence of added Ca(II). (D) CP-Ser in the presence of added Ca(II). Black circles, 0 μg/mL CP; open squares, 31.25 μg/mL CP; black diamonds, 62.5 μg/mL CP; black squares, 125 μg/mL CP; black triangles, 250 μg/mL CP; red circles, 500 μg/mL CP.

We next examined whether one or both CP metal-binding sites are required for growth inhibition of bacteria. Cultures of *S. aureus, E. coli,* and *E. aerogenes* were treated with 500 μg/mL of wild-type or mutant CP, and the growth was recorded at t = 8 and 20 h (Figure 2.3.23). The CP mutants all contain Cys → Ser mutations and do not require external reducing agents, so assays were conducted in the absence and presence of BME. A synergistic CP/BME effect was observed for *E. coli* at t = 8 h and for all bacterial species at t = 20 h, indicating that BME increases the *in vitro* antibacterial activity of CP independent
of the native cysteine residues (Figure 2.3.24). The origin of this BME effect is unclear and may be due to complexation of bioavailable Zn(II), reduction of the bacterial periplasmic Zn(II)-binding protein, reduction of other chelateable metal ions, or other perturbation of the bacteria. The CP-Ser-ΔHis₃Asp and CP-Ser-ΔHis₄ mutants both inhibited the growth of the bacterial strains tested (Figure 2.3.23); however, CP-Ser-ΔHis₄ was less effective at inhibiting bacterial growth at $t = 20$ h. At the time of this work, the origin of this effect was unclear and may point to an important role of the unusual His₄ site in host-defense. Regardless, at earlier time points ($t = 8$ h), one CP metal-binding site is necessary and sufficient for growth inhibition of several strains of bacteria.

Figure 2.3.23. Growth inhibitory activity of 500 µg/mL wild-type CP, CP-Ser, CP-Ser-ΔHis₃Asp, CP-Ser-ΔHis₄, and CP-Ser-ΔΔ. (A) Enterobacter aerogenes ATCC 13048, (B) Escherichia coli ATCC 25922, and (C) Staphylococcus aureus ATCC 25923. The cultures were incubated with 500 µg/mL CP in the presence of 2 mM Ca(II) supplement ($T = 30 \, ^{\circ}C$). The OD₆₀₀ values (mean ± SEM for three independent replicates) were recorded at $t = 8$ h (grey bars) and 20 h (white bars).
Figure 2.3.24. Synergistic effect of CP and β-mercaptoethanol (BME) on bacterial growth inhibition. Bacterial cultures were treated with 500 μg/mL CP and incubated at 30 °C with shaking in growth medium with (grey bars) or without (white bars) 3.1 mM BME (final concentration). The OD_{600} values were recorded at t = 8 h and t = 20 h. 1, wild-type CP; 2, CP-Ser, 3, ΔHis_{3}Asp; 4, ΔHis_{4}; 5, ΔΔ; 6, buffer only. (A) *E. aerogenes*, t = 8 h; (B) *E. aerogenes*, t = 20 h; (C) *E. coli*, t = 8 h; (D) *E. coli*, t = 20 h; (E) *S. aureus*, t = 8 h; (F) *S. aureus*, t = 20 h.
2.4 Summary and Perspectives

Sequestration of Zn(II) and Mn(II) from invading pathogens is one accepted mechanism of action for calprotectin in host-defense. The solution studies presented in this work reveal that both the His₃Asp and His₄ motifs contribute to the high-affinity coordination of Zn(II). Moreover, the affinities of these sites for Zn(II), as well as the antibacterial activity of CP, are modulated by Ca(II). Elucidating the molecular basis for the Ca(II)-dependent Zn(II) affinities will require further investigations. The Zn(II)-coordinating residues (A8)His27 and (A9)Asp30 reside on the Ca(II)-binding loops, and we hypothesize that Ca(II)-binding may organize the Zn(II)-coordination spheres or stabilize the Zn(II) / CP complex.

Extracellular Ca(II) concentrations are in the low-millimolar range, and intracellular levels of free Ca(II) are in the nanomolar range. We propose that CP is packaged in the neutrophil as the relatively low-affinity αβ heterodimer. When neutrophils are circulated to sites of infection, they lyse or undergo NETosis¹² to release their antibacterial contents. Upon release into the extracellular environment, CP morphs into the higher-affinity tetramer form where it competes with bacterial metal-ion transporters for Zn(II). Moreover, CP may strip Zn(II) away from Zn(II)-dependent enzymes such as microbial proteases or human matrix metalloproteinases.³⁴,³⁵ In the absence of Ca(II), site 1 of CP has a sub-nanomolar affinity for Zn(II), and it is unclear how CP may contribute to transition-metal ion homeostasis in the neutrophil cytoplasm. Neutrophils also respond to oxidized low-density lipoprotein and import Ca(II) into the cytoplasm,³⁶ and this phenomenon may have an effect on CP speciation.

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References


Chapter 3: High-Affinity Manganese Coordination by Human Calprotectin Is Calcium-Dependent and Requires the Histidine-Rich Site at the Dimer Interface

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3.1 Introduction

Calprotectin (CP) is a mammalian host-defense protein that exhibits antibacterial activity by sequestering bioavailable Zn(II) and Mn(II). Chapter 2 described the Co(II)- and Zn(II)-binding properties of human CP, and establishes that CP utilizes Ca(II)-ion gradients to tune its affinity for Zn(II) and its antibacterial activity against several species of bacteria. This chapter focuses on initial studies into Mn(II) coordination by human CP. Manganese acquisition and utilization are associated with virulence of many microbes. For example, *Staphylococcus aureus* expresses the Mn(II)-binding binding protein MntC early during infection. *Streptococcus pneumoniae* requires Mn(II) for virulence as does *Yersina pestis*. *Borrelia burgdorferi* has no apparent metabolic requirement for iron and the ZIP-family member BmtA is required for Mn(II) uptake and virulence. *Salmonella Typhimurium* encodes the Mn(II) transporters MntH and SitABCD that are required for virulence in mice. Moreover, accumulation of Mn(II) by *Neisseria gonorrhoeae* is associated with an increased resistance to oxidative killing by neutrophils.

CP was initially identified as a manganese-binding protein in a mouse model of *Staphylococcus aureus* abscesses, where Mn(II) was depleted from abscesses in an S100A9-dependent manner. Human CP exhibited growth inhibitory activity against *S. aureus* strain Newman in vitro and provided protection against Mn(II) toxicity in a strain lacking the manganese transport regulator (ΔmntR). Moreover, CP exhibited enhanced antibacterial activity against an *S. aureus* strain that was deficient in Mn(II)-uptake (ΔmntAΔmntB mutant). These studies support the notion that CP coordinates Mn(II) in the growth medium and sequesters this nutrient from *S. aureus*.

CP is the only known mammalian Mn(II)-sequestering protein, and the only S100 protein with reported Mn(II)-chelating properties. The CP heterodimer has two transition-metal-ion binding motifs at the dimer interface: a His$_3$Asp (site 1) and a His$_4$ (site 2) motif
(Figure 3.1.1). An initial ITC study supports the presence of one high-affinity Mn(II)-binding site on CP.\textsuperscript{16} This chapter describes extensive spectroscopic and metal-binding studies to decipher the Mn(II)-binding properties of CP. Analytical size exclusion chromatography provides evidence for high-affinity Mn(II) complexation at site 2, and electron paramagnetic spectroscopy demonstrates unambiguously that CP employs the His\textsubscript{4} residues, as well as two as-yet-unidentified residues to coordination Mn(II). These residues are identified and discussed further in Chapter 4. The affinity of CP for Mn(II) is Ca(II)-dependent, and CP has the ability to form mixed Ca(II) / Zn(II) / Mn(II) in which the EF-hands, His\textsubscript{3}Asp and His\textsubscript{4} site coordinate Ca, Zn, and Mn, respectively.\textsuperscript{17}

Figure 3.1.1. Structure and amino acid sequences of calprotectin (CP). (A) Depiction of a heterodimer unit of human CP (PDB ID: 1XK4). This heterodimer is taken from the crystal structure of the Ca(II)-bound heterotetramer to illustrate the metal-binding sites. No structure of the Ca(II)-free heterodimer is available. S100A8 is colored green, S100A9 is colored blue, and the Ca(II) ions are shown as yellow spheres. The His\textsubscript{3}Asp (site 1) and His\textsubscript{4} (site 2) metal-binding motifs form at the heterodimer interface, and contributing side chains are colored orange. (B) Amino acid sequence alignment of human (indicated by "h") and murine (indicated by "m") S100A8 and S100A9. The color-coded secondary structure elements correspond to the human subunits. The residues of the metal-binding motifs are colored orange. The two Cys residues that were mutated to Ser for metal-binding studies are highlighted red. The C-terminal extensions of human and murine S100A9 are underlined. The numbers indicate amino acid number.
3.2 Experimental

3.2.1 Materials and General Methods

Experimental details for the preparation of buffers for metal-binding studies, including necessary precautions to avoid metal contaminations are discussed in Chapter 2. All aqueous solutions were prepared with Milli-Q water (18.2 MΩ·cm, 0.22 µm filter). For metal-binding experiments, HEPES buffer was prepared by using metal-free Ultrol grades HEPES (free acid, Calbiochem) and TraceSELECT NaCl (Sigma), and metal-free aqueous NaOH (Sigma) was used for pH adjustments. To reduce metal-ion contamination, all buffers were treated with Chelex 100 resin (BioRad, 10 g/L) for at least 1 h prior to use. The Chelex-treated buffers were filtered through a 0.22 µm filter or centrifuged to remove Chelex resin, and stored in polypropylene containers. All metal-binding studies were conducted at pH 7.5 in 75 mM HEPES, 100 mM NaCl buffer unless otherwise specified. A Tris buffer (1 mM Tris, pH 7.5) was used for circular dichroism (CD) spectroscopy. Calcium chloride and 99.999% anhydrous zinc chloride were purchased from Sigma Aldrich, and 99.999% manganese chloride was obtained from Alfa Aesar. Stock solutions of Ca(II) (1 M), Mn(II) (1 M), and Zn(II) (100 mM) were prepared using Milli-Q water and acid-washed volumetric glassware, and the solutions were immediately transferred to and stored in polypropylene containers. Each working M(II) solution was made fresh daily by diluting the appropriate stock solution to the desired working concentration with either Milli-Q water or metal-free buffer. Zinpyr-1 (ZP1) was purchased from Strem Chemicals Co., and the purity was verified by analytical HPLC analysis, or synthesized from 2',7'-dichlorofluorescin and di(2-picoyl)amine as described elsewhere. Stock solutions of ZP1 (ca. 2 mM) were prepared in anhydrous DMSO, partitioned into 50-µL aliquots, and stored at -20 °C. Each aliquot was freeze-thawed only once, and the working ZP1 concentration was verified by using the reported extinction coefficient of apo ZP1 ($\varepsilon_{515} = 79,000 \text{ M}^{-1} \text{ cm}^{-1}$).
3.2.2 Preparation of Mutant CP and Design and Purification of CP-Ser(H27D)

Recombinant human CP and mutant proteins were overexpressed, purified, and characterized as described in Chapter 2 and reported elsewhere. The metal-binding studies presented in this chapter were conducted by using CP-Ser and mutants thereof. CP-Ser is comprised of the subunits S100A8(C42S) and S100A9(C3S). These Cys-to-Ser mutants were employed to avoid the need for reducing agents in the metal-binding studies. The mutated cysteine residues are not essential for the antibacterial activity of CP. CP-Ser, CP-Ser-ΔHis$_3$Asp, CP-Ser-ΔHis$_4$, CP-Ser-ΔΔ, and CP-Ser(H27D) (vide infra) were prepared multiple times, and results from independent batches of protein were comparable. CP-Ser(H17A), CP-Ser(H27A), CP-Ser(H91A), CP-Ser(H27A)(H91A) were each prepared and purified once. Wild-type CP was also purified once and was used only for analytical size exclusion chromatography experiments.

We generated and reconstituted a mutant CP-Ser comprised of S100A8(C42S)(H27D) and S100A9(C3S) to investigate the importance of the His$_4$ motif at site 2. Mutation of (S100A8)His27 to Asp was selected based on sequence alignments of S100A8 with other transition-metal binding S100 proteins including S100A7, S100A9, and S100A12. A modified quick-change mutagenesis protocol (Strategene) was employed for site-directed mutagenesis as described in Chapter 2. The template plasmid pET41a-S100A8(C42S) contains an E. coli codon-optimized synthetic gene for S100A8(C42S) inserted between Ndel and Xhol restriction sites of pET41a. The primers 5’-caaggtaactttgacgcgtctatgtg-3’ and 5’-gttcccattgaactgctgccagatgcag-3’ (mutation site underlined) provided the H27D mutation. The PCR protocol employed for the mutagenesis reaction was: 95 °C for 30 min; 55 °C for 1 min, 68 °C for 17 min (25x); 4 °C hold. PfuTurbo DNA polymerase was employed in the PCR reaction. Following PCR amplification, the template plasmid was digested with DpnI (New England Biolabs; 1.5 μL added to a 25-μL PCR reaction in two 0.75-μL aliquots at t = 0 and 1.5 h) for 3 h at 37 °C. The DpnI digests
were transformed into chemically-competent *E. coli* TOP10 cells. Overnight cultures (5 mL, 50 µg/mL kanamycin) were grown from single colonies, and a miniprep kit (Qiagen) was employed to obtain purified plasmids. The DNA sequences and presence of the H27D mutation were verified by DNA sequencing (MIT Biopolymers Facility). The resulting pET41a-S100A8(C42S)(H27D) plasmid was transformed into chemically-competent *E. coli* BL21(DE3) cells. Overexpression of S100A8(C42S)(H27D) and reconstitution with S100A9(C3S) to generate the CP-Ser(H27D) mutant was performed as described in Chapter 2. Purified CP-Ser(H27D) was obtained in yields ranging from 25–65 mg / 4 L of culture. The protein was characterized by SDS-PAGE, mass spectrometry, CD spectroscopy, and analytical gel filtration chromatography as previously described.\(^{17}\)

Protein concentrations were routinely determined by using a calculated coefficient for the CP heterodimer (\(\varepsilon_{280} = 18,450 \text{ M}^{-1} \text{ cm}^{-1}\) for CP and all mutants discussed). A BioTek Synergy HT plate reader outfitted with a calibrated Take3 micro-volume plate, or an Agilent 8453 diode array spectrophotometer, was routinely employed to determine protein concentrations.

### 3.2.3 Analytical Size Exclusion Chromatography

CP-Ser and metal-binding site mutants were buffer exchanged into Chelex-treated buffer at pH 7.5 (75 mM HEPES, 100 mM NaCl). The buffer for wild-type CP contained 1 mM β-mercaptoethanol to prevent disulfide formation. The protein samples were adjusted to a concentration of 200 µM, and 10 equivalents of Mn(II) (14 µL from a 100 mM stock) was added to a final volume of 700 µL. The samples were incubated on ice or in a cold room (4 °C) for 0, 2, or 8-18 h. At each time point, a 200-µL aliquot was loaded onto a Superdex 75 10/300 GL (GE Lifesciences) size-exclusion column via a 500-µL sample loop connected to an ÄKTA Purifier FPLC system (GE Lifesciences). The column was pre-equilibrated with 1.5 column volumes of running buffer (Chelex-treated 75 mM HEPES, 100 mM NaCl, pH 7.5),
and the sample loop was emptied with 0.5 mL of running buffer. Details for the calibration of the S75 column are provided in Chapter 2. The sample was subsequently eluted over 1 column volume at a flow rate of 0.5 mL / min at room temperature. The protein-containing fractions, identified by monitoring absorption at 280 nm, were collected, and protein concentration of each fraction was quantified by using the molar extinction coefficient at 280 nm ($\epsilon_{280} = 18,450 \, M^{-1} \, cm^{-1}$).

### 3.2.4 Manganese Quantification by Atomic Absorption Spectroscopy

The manganese content of each protein-containing fraction obtained from analytical size exclusion chromatography (SEC) was determined by atomic absorption spectroscopy. A Perkin-Elmer AAnalyst 600 atomic absorption spectrometer was employed for all measurements. The protein-containing fractions were diluted 1:200 into Milli-Q water. A Mn(II) standard curve (0 – 5 µg/L) was obtained by using solutions of Mn(II) (1000 µg/mL MnCl₂ atomic absorption standard, J.T. Baker) dissolved in 5% nitric acid and diluted with Milli-Q water using acid-washed volumetric glassware. The averages obtained from three independent samples are reported, and the errors are the standard deviation from the mean.

### 3.2.5 Optical Absorption and Fluorescence Spectroscopy

Optical absorption spectra were recorded on either a Beckman Coulter (DU800) scanning spectrophotometer or an Agilent 8453 diode array UV-visible spectrophotometer thermostatted at 25 °C. Fluorescence spectra were collected on a Photon Technologies International QuantaMaster 40 fluorimeter outfitted with a continuous xenon source for excitation, autocalibrated QuadraScopic monochromators, a multimode PMT detector, and a circulating water bath maintained at 25 °C. The spectrofluorimeter was controlled by the FelixGX software package. Quartz cuvettes (Starna) with a 1-cm path length were employed for all optical absorption and fluorescence measurements. To prevent metal-ion
contamination, the cuvettes were washed with 20% nitric acid and thoroughly rinsed with Milli-Q water prior to use. The cuvettes were stored in 20% nitric acid.

### 3.2.6 Circular Dichroism Spectroscopy

Protein solutions (10 μM, 300 μL) were prepared at pH 7.5 in 1.0 mM Tris buffer that was treated with Chelex to remove trace metals. The pH was readjusted to 7.5 by addition of hydrochloric acid after the Chelex treatment. For Mn(II)-binding experiments, the CD spectrum of the apo protein was recorded. Subsequently, Mn(II) was added to a final concentration of 100 μM, the sample was mixed gently, and the CD spectrum was recorded ca. 2 min after Mn(II) addition. This experiment was also conducted in the presence of 2 mM Ca(II). The CD spectra were collected from 260 – 195 nm at 1 nm intervals (3 sec averaging time, three independent scans per wavelength). The data obtained from the three scans were averaged by using Excel and plotted in KaleidaGraph. For thermal denaturation experiments with mutant proteins, a sample of 10 μM CP-Ser-ΔHis<sub>3</sub>Asp or CP-Ser-ΔHis<sub>4</sub> was prepared at pH 8.5 (1 mM Tris, 0.5 mM EDTA), and the temperature was varied from 25 – 95 °C in 2 °C increments. Samples containing Ca(II) were prepared in the same manner except that Ca(II) was added to a final concentration of 2 mM. These conditions were selected based on prior literature studies that reported CD spectra of wild-type CP in the absence and presence of Ca(II) under such buffer conditions. For thermal denaturation of CP-Ser in the presence of Mn(II), 10 μM CP-Ser was prepared at pH 7.5 (1.0 mM Tris, Chelex-treated) and incubated with 10 equivalents of Mn(II) in the absence and presence of 2 mM Ca(II). For all thermal denaturation experiments, the CD signal at 222 nm was recorded at each temperature after one-minute equilibration time. A 1-mm path-length quartz CD cell (Hellma) was employed for all CD measurements.
3.2.7 General Methods for Electron Paramagnetic Resonance Spectroscopy

Electron paramagnetic resonance (EPR) spectra (X-band, 9 GHz) were recorded on a Bruker EMX spectrometer outfitted with an ER 4199HS cavity. A flat quartz cell (Bruker Biospin) positioned in an E-field null plane within the cavity was used for all room-temperature measurements. An ESR900 cryostat was used for all low-temperature measurements, and the temperature was monitored with either a Cernox sensor or a thermocouple. A copper-EDTA spin standard was used to account for all relevant intensity factors. All spectral analysis and simulations were performed with the Windows software package, SpinCount, developed by Professor Michael P. Hendrich at Carnegie Mellon University. The simulations of protein-bound Mn(II) were determined using the standard spin Hamiltonian described by equation 1 with second-order perturbation theory.

\[ H_s = D \left( S_z^2 - \frac{(S(S+1))}{3} \right) + E(S_X^2 + S_Y^2) + \beta B g S + S_A I \]  

\( S \) is the spin of the system, \( \beta \) is the Bohr magneton, \( B \) is the magnetic field strength, \( D \) and \( E \) are the zero-field splitting parameters, \( I \) is the nuclear spin, and \( g \) is the g-tensor. SpinCount treats nuclear hyperfine interactions (\( A \)) with second-order perturbation theory.

3.2.8 Room-Temperature EPR Spectroscopy

In a typical room-temperature EPR titration, a 300-\( \mu \)L sample of CP-Ser (ca. 25 or 150 \( \mu \)M for titrations in the presence or in the absence of Ca(II), respectively) was prepared at pH 7.5 (75 mM HEPES, 100 mM NaCl) and titrated with a Mn(II) solution freshly prepared in the buffer. The sample was incubated for at least 10 minutes at room temperature after each Mn(II) addition and placed in a flat quartz cell. The EPR spectrum was recorded using the following conditions: microwaves, 9.8 GHz, 20 mW; modulation, 1.0 mT. The concentration of free Mn(II) in each sample was determined by scaling the inner four lines of the EPR spectrum to those of the standard Mn(II) solution prepared from an atomic
absorption standard (J.T. Baker) diluted in the buffer and recorded under identical conditions. The Mn(II) standard was also used to verify the concentration of the Mn(II) stock solution employed for the metal-binding titration. Room-temperature titrations for the heterodimeric single-point mutants CP-Ser(H17A), CP-Ser(H27A), CP-Ser(H27D), CP-Ser(H91A), CP-Ser(H95A), the double-point mutant CP-Ser(H27A)(H91A), and CP-Ser-ΔΔ in the absence and presence of Ca(II) were each performed twice. Titrations of CP-Ser, CP-Ser-ΔHis₃Asp, and CP-Ser-ΔHis₄ were performed at least in triplicate. Titrations of CP-Ser in the presence of Ca(II) were performed in duplicate. The Mn(II) dissociation constant values obtained from room-temperature EPR titrations of CP-Ser were obtained from global fits of the data obtained from multiple titrations. The equations employed to fit the binding curves are given below for the absence of Ca(II) (equation 2) and presence of Ca(II) (equation 3).²⁰

\[
\frac{[\text{Mn}_{\text{bound}}]}{[\text{CP}]} = \left( \frac{[\text{Mn}_{\text{free}}]}{(K_{d1}+[\text{Mn}_{\text{free}}])} \right) + \left( \frac{[\text{Mn}_{\text{free}}]^2}{(K_{d2}+[\text{Mn}_{\text{free}}]^2)} \right) \quad (2)
\]

\[
\frac{[\text{Mn}_{\text{bound}}]}{[\text{CP}]} = \left( \frac{[\text{Mn}_{\text{free}}]}{(K_{d1}+[\text{Mn}_{\text{free}}])} \right) + \left( \frac{[\text{Mn}_{\text{free}}]}{(K_{d2}+[\text{Mn}_{\text{free}}])} \right) \quad (3)
\]

3.2.9 Low-Temperature EPR Spectroscopy

Low-temperature EPR samples were housed in 4-mm (outer diameter) quartz EPR tubes and frozen in liquid nitrogen prior to analysis. Low-temperature EPR titrations were performed with 200-µL samples of CP at the indicated concentrations. Aliquots of aqueous Mn(II) were added directly into the EPR tube containing a solution of the protein by using a 100-µL Hamilton syringe. The sample was then mixed with a second 250-µL Hamilton syringe and incubated for a minimum of 10 min at room temperature before being frozen in liquid nitrogen. The EPR spectra of CP samples containing Mn(II) were unperturbed by
repeated freeze-thaw cycling. Low-temperature spectra of Mn(II):CP mixtures in the presence or in the absence of 2 mM Ca(II) or 600 mM NaCl were also recorded. For these spectral acquisitions, samples containing protein and Mn(II) were thawed, and either NaCl or CaCl₂ was added from a concentrated stock solution (typically >10% dilution of the original sample). The sample was allowed to incubate for a minimum of 15 min at room temperature before being frozen in liquid nitrogen.

Low-temperature Mn(II)-binding EPR titrations were performed in the absence and in the presence of Ca(II). For the titrations conducted in the absence of Ca(II), the CP-Ser concentration was ca. 100 μM, and 0 – 1.1 equivalents of Mn(II) were added. For the titrations in the presence of Ca(II), CP-Ser (ca. 100 μM) was incubated with 1 mM of CaCl₂ for 15 min or more prior to titrating the sample with Mn(II). The resulting spectra were analyzed for the formation and growth of distinct Mn(II)-bound signals with respect to the concentration of Mn(II) added. For this analysis, the intensity of the highest field hyperfine resonance, defined as the change in the y-axis from the positive feature at $g = 1.88$ to the negative feature at $g = 1.873$, was determined for each titration point. The intensity of this feature was correlated to the concentration of CP-bound Mn(II) by defining the intensity from a sample of CP-Ser with 0.1 equivalents of Mn(II) in the presence of Ca(II) as being equal to the concentration of metal ion added (ca. 10 μM, assuming that all added metal becomes bound metal under these conditions). The concentration of CP-bound Mn(II) for all titrations was determined using this ratio. Each titration was repeated twice, and the Mn(II) dissociation constants were determined from a global fit of the combined data.

3.2.10 Mn(II) Competition Experiments with ZP1

For competition experiments between CP-Ser and ZP1, a 2-mL solution of ZP1 (1–4 μM) was prepared in a quartz cuvette at pH 7.5 (75 mM HEPES, 100 mM NaCl). The optical absorption and fluorescence spectra of ZP1 were recorded. An aliquot of CP was introduced
to the cuvette to afford the desired protein concentration (1–4 μM), and the optical and fluorescence spectra were recorded again. If metal-ion contamination was detected, indicated by a change in ZP1 emission relative to the protein spectrum, the solution was discarded. For manganese competition experiments, aliquots of aqueous Mn(II) from a freshly prepared 0.5 or 1 mM Mn(II) working solution were added to the ZP1/CP mixture. Following each Mn(II) addition, the solution was incubated in the dark for 10 min at room temperature, and the optical absorption and emission spectra were subsequently recorded. The same procedure was also utilized for experiments where Ca(II) was added to mixtures containing CP-Ser / ZP1 / Mn(II) except that the sample was incubated for 15 min at room temperature after each Ca(II) addition. The emission spectra were recorded from 500 – 650 nm (λ<sub>ex</sub> = 490 nm, 0.4 nm excitation and emission slit widths) and integrated over this range by using the FelixGX software package.
3.3. Results and Discussion

3.3.1 Analytical SEC Reveals that the His\textsubscript{4} Site Formed at the CP (αβ) Dimer Interface is Required for Mn(II) Coordination

We first performed analytical SEC to ascertain whether Mn(II) was retained by CP after chromatography and to determine whether the presence of Mn(II) causes a shift in the elution volume of CP. We have previously established that in the absence of added Ca(II), the CP heterodimer elutes after ca. 10.8 mL (ca. 38 kDa). When 2 mM Ca(II) is included in the running buffer, the CP heterotetramer has an elution volume of ca. 10.2 mL (ca. 48 kDa). Ca(II)-dependent tetramer formation is seen with wild-type CP, CP-Ser, CP-Ser-ΔHis\textsubscript{3}Asp, CP-Ser-ΔHis\textsubscript{4}, and CP-Ser-ΔΔ (Appendix 2). CP and CP-Ser were incubated with 10 equivalents of Mn(II), and the resulting mixture was eluted at pH 7.5 (75 mM HEPES, 100 mM NaCl). A new Mn(II)-dependent peak was observed for CP and CP-Ser, with an elution volume of ca. 10.3 mL (Figure 3.3.1, Table 3.3.1). This Mn(II)-dependent shift was also observed for the ΔHis\textsubscript{3}Asp mutant, but not the ΔHis\textsubscript{4} or ΔΔ mutants. These results demonstrate that the formation of the Mn(II)-dependent peak at 10.3 mL requires the His\textsubscript{4} motif.

Following elution, the protein-containing fractions were analyzed for Mn content by atomic absorption spectroscopy. This analysis revealed that Mn(II) was retained only by CP, CP-Ser, and CP-Ser-ΔHis\textsubscript{3}Asp. The Mn(II) / CP ratios spanned 0.4-0.7 for these samples (Table 3.3.1). In contrast, negligible Mn(II) was retained in the CP-Ser-ΔHis\textsubscript{4} and CP-Ser-ΔΔ samples. These experiments suggest that CP coordinates Mn(II) at site 2, and the affinity is sufficient to retain Mn(II) over the course of the column. Furthermore, the Mn(II)-dependent shift in the elution volume for CP, CP-Ser, and CP-Ser-ΔHis\textsubscript{3}Asp indicates that Mn(II)-complexation by CP results in a conformational change.
Figure 3.3.1. Analytical SEC of CP and mutant proteins in the absence and presence of 10 equivalents of Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). (A) CP-Ser. (B) CP-Ser with 2 mM Ca(II) in the running buffer. (C) Wild-type CP pre-incubated with 10 equivalents of Mn(II). (D) CP-Ser pre-incubated with 10 equivalents of Mn(II). (E) CP-Ser-ΔHis$_3$Asp pre-incubated with 10 equivalents of Mn(II). (F) CP-Ser-ΔHis$_4$ pre-incubated with 10 equivalents of Mn(II). (G) CP-Ser-ΔΔ pre-incubated with 10 equivalents of Mn(II). The chromatograms in A and B contained 100 μM CP and were scaled 2x. The vertical dashed lines indicate the elution volumes for the CP-Ser αβ and α$_2$β$_2$ forms.

### Table 3.3.1. Mn(II) Quantification of Protein-Containing SEC Samples.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Elution Volume (mL)</th>
<th>Mn/CP$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td>10.3</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>CP-Ser</td>
<td>10.3</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>ΔHis$_3$Asp</td>
<td>10.3</td>
<td>0.70 ± 0.14</td>
</tr>
<tr>
<td>ΔHis$_4$</td>
<td>10.9</td>
<td>0.012 ± 0.003</td>
</tr>
<tr>
<td>ΔΔ</td>
<td>10.9</td>
<td>0.005 ± 0.002</td>
</tr>
</tbody>
</table>

$^a$Protein concentrations were determined by absorbance at 280 nm, and the Mn(II) concentrations were quantified by atomic absorption spectroscopy. The Mn/CP ratios are per CP heterodimer.

The CD spectra of CP-Ser (±Ca) in the absence and presence of Mn(II) were unperturbed, and the minima at 222 and 208 nm confirm that the Mn(II)-bound form of CP remains α-helical (Figure 3.3.2). We conclude that coordination of Mn(II) by CP does not perturb the secondary structure of CP, and that this is not the cause of the change in elution
volume observed by SEC. We hypothesize that the tertiary or quaternary structure of CP is modulated by the addition of Mn(II) ions.

![Circular dichroism spectra](image)

**Figure 3.3.2.** Circular dichroism spectra of CP-Ser (10 µM) in the absence and presence of 100 µM Mn(II) at pH 7.5 (1 mM Tris). The black line is the CD spectrum in the absence of any metal ions. The blue line is the CD spectrum of CP-Ser in the presence of 10 equivalents of Mn(II), and the red line is the CD spectrum in the presence of Mn(II) and 2 mM Ca(II).

We next examined whether mutation of the transition metal-ion coordinating residues to alanine, or coordination of Mn(II), affects the thermal stability of CP-Ser. The CP-Ser-\Delta His$_3$Asp and CP-Ser-\Delta His$_4$ mutants display $T_m$ values similar to CP-Ser in the absence and presence of Ca(II) (Figure 3.3.3 panel A). In the absence of Ca(II), these proteins exhibit melting temperatures from ca. 55-59 °C. Ca(II) ions stabilize the protein fold to thermal denaturation, and the $T_m$ values are in the range of 75-79 °C. The alanine mutations do not affect the thermal stability of CP.

Addition of Mn(II) to CP-Ser at pH 7.5 (1 mM Tris) increased the thermal stability to ca. 88 °C, and the addition of Ca(II) and Mn(II) precluded unfolding up to 95 °C (Figure 3.3.3 panel B). Manganese coordination increases the stability of CP-Ser, and the Mn(II)-bound
form is stable at higher temperatures. Moreover, melting of the Ca(II)- and Mn(II)-bound form of CP was not observed up to 95 °C, indicating that this complex is very stable to thermal denaturation.

**Figure 3.3.3.** Thermal denaturation of CP-Ser and mutants. (A) Thermal denaturation of CP-Ser (black), ΔHis₃Asp (blue), and ΔHis₄ (red) at pH 8.5 (1 mM Tris, 0.5 mM EDTA) in the absence (circles) and presence (squares) of 2 mM Ca(II). The CP-Ser data was taken from chapter 2. (B) Thermal denaturation of CP-Ser in the absence of metals (black circles) and in the presence of 2 mM Ca(II) (blue squares), 100 μM Mn(II) (green triangles), or 100 μM Mn(II) and 2 mM Ca(II) (red diamonds) at pH 7.5 (1 mM Tris). The protein concentration was 10 μM for all experiments.

### 3.3.2 Room-Temperature Mn(II) EPR Titrations Confirm the Requirement of the His₄ Site

We employed room-temperature EPR spectroscopy to further investigate the Mn(II)-binding properties of CP-Ser. High-spin Mn(II) is an $S = 5/2$ system that is readily observed by EPR spectroscopy. At room temperature, $[\text{Mn(H}_2\text{O)}_6]^{2+}$ exhibits a six-line pattern at $g = 2$ that results from hyperfine splitting of the allowed EPR transition ($\Delta m_s = \pm 1, \Delta m_I = 0$) with the $^{55}\text{Mn}$ nucleus ($I = 5/2$). Coordination of Mn(II) by proteins typically broadens the zero-field splittings of high-spin Mn(II) beyond detection at room temperature, and cryogenic temperatures are employed to observe such signals (*vide infra*). CP-Ser and the metal-
binding site mutants were titrated with 0-4 equivalents of Mn(II) / dimer at pH 7.5 (75 mM HEPES, 100 mM NaCl), and the room-temperature EPR spectrum of each sample was recorded. Each Mn(II)-containing sample exhibited a six-line pattern with a splitting of a = 8.9 mT and centered at g = 2. Only the intensity of the spectra varied throughout each titration, supporting the notion that only the manganese(II) hexaaqua signal is observed. The [Mn(II)] in each sample was quantified by comparing the signal intensity of the inner four lines to a Mn(II) standard recorded under identical conditions. Plots of [Mn(II)] / CP vs. [Mn(II)] / CP reveal an attenuation of the [Mn(H2O)6]2+ signal when the His4 site is present. No attenuation of the signal was observed for the CP-Ser-ΔHis4 or CP-Ser-ΔΔ mutant, and the [Mn(II)] grew linearly with added Mn(II) (slope ≈ 1, Figures 3.3.4 and 3.3.5, Table 3.3.2). These titrations support the formation of a Mn(II) / CP complex and indicate that at least some of the residues of the His4 motif are required for Mn(II) coordination.

![Figure 3.3.4](image)

**Figure 3.3.4.** Plots of [Mn(II)] / CP versus [Mn(II)] / CP obtained from room-temperature Mn(II) EPR titrations of 149 μM CP-Ser (red circles), 132 μM CP-Ser-ΔHis3Asp (blue circles), 127 μM CP-Ser-ΔHis4 (black squares), and 165 μM CP-Ser-ΔΔ (black diamonds) at pH 7.5 (75 mM HEPES, 100 mM NaCl).

We next titrated Mn(II) into samples of CP-Ser(H17A), CP-Ser(H27A), CP-Ser(H91A), CP-Ser(H95A), and the double mutant CP-Ser(H27A)(H91A). In all of these cases, the plots of [Mn(II)] / CP vs. [Mn(II)] / CP revealed linear increases in the concentration of free Mn(II) (Figure 3.3.5 and Table 3.3.2). With the exception of the CP-
Ser(H17A), which afforded slopes ≥ 1, the single- and double-mutant data afforded slopes spanning 0.72-0.92. These results indicate that (A8)His17, (A8)His27, (A9)His91, and (A9)His95 are important for Mn(II) coordination by CP-Ser.

Figure 3.3.5. Analysis of the data obtained from Mn(II) room-temperature EPR titrations for CP-Ser mutants. Plots of [Mn(II)\text{free}] versus [Mn(II)\text{total}] for the CP-Ser mutants H17A, H27A, H27D, H91A, (H17A)(H91A), H95A, ΔHis4, ΔA, and ΔA in the presence of 500 μM Ca(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). In one set of titrations, the protein concentration was ~ 100 μM for H17A, H27A, H27D, H91A, H95A, and ΔA. In a second set of titrations, the protein concentration was ca. 30 μM for these mutants. Both ΔHis4 titrations were conducted with ca. 100 μM protein. Both ΔA + Ca(II) titrations were conducted with ca. 30 μM protein and 500 μM Ca(II). The titration of Mn(II) into buffer is not shown.
The His₄ motif of CP is unique among transition-metal ion coordinating S100 proteins. Alignment of the sequences of S100A8 and S100A9 with S100A7 and S100A12 reveals that S100A8 contains a unique His residue at position 27. The other S100 proteins house a conserved Asp residue. To further evaluate the importance of this residue, we employed site-directed mutagenesis to generate an (A8)H27D mutant. Titration of CP-Ser(H27D) with 0-3 equivalents of Mn(II) affords a linear relationship (slope = 0.7-0.8), indicating that Mn(II) coordination is attenuated relative to CP-Ser (Figure 3.3.5 and Table 3.3.2). This result further supports the notion that the unusual His₄ motif is necessary for Mn(II) coordination by CP.

### Table 3.3.2. Slope Analysis of Room-Temperature EPR Titrations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Slope</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>1.00</td>
<td>0.9967</td>
</tr>
<tr>
<td>CPSer(H17A)</td>
<td>1.3466</td>
<td>0.9787</td>
</tr>
<tr>
<td>CPSer(H27A)</td>
<td>0.989</td>
<td>0.9902</td>
</tr>
<tr>
<td>CPSer(H27A)</td>
<td>0.8228</td>
<td>0.9948</td>
</tr>
<tr>
<td>CPSer(H27A)</td>
<td>0.7251</td>
<td>0.9902</td>
</tr>
<tr>
<td>CPSer(H27D)</td>
<td>0.7827</td>
<td>0.9949</td>
</tr>
<tr>
<td>CPSer(H27D)</td>
<td>0.6865</td>
<td>0.9829</td>
</tr>
<tr>
<td>CPSer(H91A)</td>
<td>0.8547</td>
<td>0.9948</td>
</tr>
<tr>
<td>CPSer(H95A)</td>
<td>0.7376</td>
<td>0.9929</td>
</tr>
<tr>
<td>CPSer(H95A)</td>
<td>0.7376</td>
<td>0.9947</td>
</tr>
<tr>
<td>CPSer(H91A)</td>
<td>0.9223</td>
<td>0.988</td>
</tr>
<tr>
<td>CPSer(H17A)(H91A)</td>
<td>0.8395</td>
<td>0.9964</td>
</tr>
<tr>
<td>CPSer ΔHis₄</td>
<td>0.8294</td>
<td>0.9938</td>
</tr>
<tr>
<td>CPSer ΔHis₄</td>
<td>1.0452</td>
<td>0.9957</td>
</tr>
<tr>
<td>CPSer ΔΔ</td>
<td>1.1266</td>
<td>0.9885</td>
</tr>
<tr>
<td>CPSer ΔΔ + Ca(II)</td>
<td>0.8596</td>
<td>0.9901</td>
</tr>
<tr>
<td>CPSer ΔΔ + Ca(II)</td>
<td>0.7384</td>
<td>0.9581</td>
</tr>
</tbody>
</table>

*The slopes were obtained from linear fits of the plots of [Mn(II)ₜᵢᵢᵢ] versus [Mn(II)ₜₒᵢᵢᵢ]. The plots are provided in Figure 3.3.5. Each entry represents an independent titration.*

### 3.3.3 The Mn(II) Affinity of the His₄ Site is Modulated by Calcium Ions

The room-temperature Mn(II) titrations monitored by EPR spectroscopy were used for $K_d$ determination in the absence and presence of Ca(II) (Figure 3.3.6). In the absence of Ca(II), a $K_{d,site2}$ value of 4.9 ± 1.0 μM was assigned to the His₄ site. Fitting of this titration
curve required the incorporation of two low-affinity Mn(II) binding events which were fixed with dissociation constants of $K_d = 1.0 \text{ mM}$. These low-affinity binding events may originate from weak Mn(II) associate with the EF-hand domains, the His$_3$Asp motif, or other acidic residues (see Appendix 4 for further discussion). Regardless, it is unlikely that these millimolar binding events are physiologically relevant or contribute to Mn(II)-sequestration by CP.

![Figure 3.3.6](image)

**Figure 3.3.6.** Dissociation constant plots obtained from room-temperature Mn(II) EPR titrations at pH 7.5 (75 mM HEPES, 100 mM NaCl). (A) Titration of ca. 100 µM CP-Ser ($\alpha_1\beta_1$) with Mn(II) afforded $K_{d1} = 4.9 \pm 1.0 \mu\text{M}$ and $K_{d2} = 1.0 \text{ mM}$ ($n = 2$, fixed). (B) Titration of 25 µM CP-Ser in the presence of 1.0 mM Ca(II) afforded $K_{d1} = 194 \pm 203 \text{ nM}$ and $K_{d2} = 21 \pm 5 \mu\text{M}$. The circles, squares, triangles, diamonds, and crosses indicate independent titrations. The lines represent the global fits of all data.

The affinity of CP-Ser for Zn(II) is modulated by Ca(II) coordination by the EF-hand domains (Chapter 2), and we reasoned that the affinity of CP for Mn(II) may also exhibit Ca(II)-dependence. Titration of Mn(II) into a mixture of Ca(II) and CP-Ser ($\alpha_2\beta_2$) at pH 7.5 (75 mM HEPES, 100 mM NaCl) revealed almost no detectable growth of the [Mn(H$_2$O)$_6$]$^{2+}$ signal until > 1 equivalent of Mn(II) / CP ($\alpha_1\beta_1$) had been added. This stoichiometric response indicates that CP-Ser has a high affinity for Mn(II). Micromolar levels of protein are required to observe any signal by room-temperature EPR methods, and the low amounts of observable Mn(II) complicate $K_d$ determination. Nevertheless the resulting binding curve
yielded $K_{d,site2} = 194 \pm 203 \text{nM}$ that we assign to the His$_4$ site. A second-binding event was required to fit the data and afforded $K_{d,site1} = 21 \pm 5 \mu\text{M}$. We assign this binding event to Mn(II) coordination by the His$_3$Asp site.

Because stoichiometric binding of Mn(II) was observed in the presence of Ca(II), we employed Mn(II) competition assays to verify the Ca(II)-effect on Mn(II) affinity and to gain further insight into the Ca(II)-dependent $K_d$. We selected ZP1, a Ca(II)-insensitive fluorescent molecule, for these studies. ZP1 is typically used as a turn-on Zn(II) sensor; however, apo ZP1 exhibits relatively high fluorescence, and this fluorescence is quenched by paramagnetic metal ions such as Mn(II). Prior studies indicate that Mn(II) coordination induces a ca. 8-fold decrease in fluorescence, and that ZP1 coordinates two equivalents of Mn(II) with apparent $K_{d1} = 550 \text{nM}$ and apparent $K_{d2} = 2.2 \mu\text{M}$ at pH 7.0 (50 mM PIPES, 100 mM KCl).$^{21}$ In particular, the 550 nM binding event indicates that ZP1 may be a useful tool to study Mn(II) coordination by CP-Ser.

Titration of a 1:1 mixture of CP-Ser:ZP1 in the absence of Ca(II) resulted in a fluorescence response comparable to the ZP1-only sample, which demonstrates that the CP-Ser heterodimer cannot compete with ZP1 for Mn(II) under these conditions (Figure 3.3.7). This result is in general agreement with the room temperature EPR titrations. In contrast, titration of Mn(II) into a sample containing 4 µM CP-Ser, 4 µM ZP1, and 200 µM Ca(II) resulted in attenuation of the ZP1 response (Figure 3.3.7). A decrease in fluorescence was observed only following the addition of >1 equivalent of Mn(II) / CP heterodimer; ZP1 cannot compete with the CP tetramer for Mn(II). ZP1 was unable to compete with the CP-Ser-ΔHis$_3$Asp tetramer for Mn(II), and ZP1 outcompeted the CP-Ser-ΔHis$_4$ tetramer for Mn(II). These data support the notions that the His$_4$ motif is required for high-affinity Mn(II) coordination, and the affinity of the His$_4$ motif for Mn(II) is modulated by Ca(II) ions. We contend that Ca(II) coordination by the EF-hands triggers high-affinity Mn(II) chelation by CP. The ZP1 competition titrations presented here support a Mn(II) $K_d$ value for the His$_4$ site.

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that switches from >550 nM to <550 nM with Ca(II) and agree with the room-temperature EPR titrations.

To evaluate how many equivalents of Ca(II) / CP are required to sequester Mn(II) from ZP1, mixtures containing a 4:4:1 ratio of CP-Ser, Mn(II), and ZP1 were titrated with Ca(II). Maximum fluorescence increase, indicating full dissociation of Mn(II) from ZP1, was observed with the addition of approximately 20 equivalents of Ca(II) / CP dimer. CP houses four EF-hand domains, and the affinity of each EF-hand for Ca(II) is unknown.

![Graph](image)

**Figure 3.3.7.** Competition between ZP1 and CP for Mn(II) in the absence and presence of Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl) and T = 25 °C. (A) Titration of a 1:1 ratio of ca. 4 μM ZP1 and 4 μM CP with Mn(II) in the absence or presence of 200 μM Ca(II). Black diamonds, ZP1 only; red circles, CP-Ser without Ca(II); red squares, CP-Ser with 50 equivalents of Ca(II); blue squares, CP-Ser-ΔHis3Asp with 50 equivalents of Ca(II); black squares, CP-Ser-ΔHis4 with 50 equivalents of Ca(II). (B) Titration of a mixture of 4 μM CP-Ser, 4 μM Mn(II), and 1 μM ZP1 with Ca(II). Excitation was provided at 490 nm, and the emission spectra were integrated from 500 – 650 nm and normalized with respect to apo ZP1 emission (A) or maximum ZP1 emission (B).
3.3.4 Low-Temperature Mn(II) EPR Spectroscopy Supports Octahedral Coordination at the His₄ Site

With the analytical SEC, room-temperature EPR, and ZP1 competition assays demonstrating that the unusual His₄ site is responsible for high-affinity Mn(II) coordination, we employed low-temperature EPR spectroscopy to identify spectroscopic signals for the Mn(II) / CP-Ser complex. Samples containing ca. 100 μM CP-Ser or mutant were incubated with 0.3 equivalents of Mn(II) for 15 min and frozen in liquid nitrogen prior to low-temperature spectroscopic analysis. All spectra are dominated by a six-line pattern at $g = 2$ with splitting of $a = 8.9$ mT as expected for high-spin $^{55}$Mn(II) systems, and all signals exhibited Curie-Law dependence (Figure 3.3.8 panel A). No significant signal intensity away from $g = 2$, as has been observed for other Mn(II)-binding proteins, was observed and indicated that the zero-field splittings for Mn(II)-CP are relatively small. The low-temperature EPR signal resulting from Mn(II) binding to CP-Ser and CP-Ser-ΔHis₃Asp were identical, indicating that the His₃Asp site does not coordinate Mn(II) under these conditions. In contrast, the spectra for Mn(II) with CP-Ser-ΔHis₄ and CP-Ser-ΔΔ were broader, less intense, and closely resembled the Mn(II) in buffer sample. The sharper lines observed for Mn(II) addition to CP-Ser and CP-Ser-ΔHis₃Asp are attributed to a more defined ligand field for Mn(II) coordinated at site 2. Similar line sharpening was observed for Mn(II) binding to the hammerhead ribozyme.²² Moreover, there is a slight shift in the positions of the allowed resonance from CP-Ser and CP-Ser-ΔHis₃Asp relative to CP-Ser-ΔHis₄ and CP-Ser-ΔΔ. The low-field transition shifts by ca. 1.5 mT, and the first allowed transition of the CP-Ser sample exhibits a shoulder at $g = 2.17$, which corresponds to the first allowed transition in CP-Ser-ΔHis₄ and CP-Ser-ΔΔ. Mutation of any single His₄ residue to Ala, or His27 to Asp, also results in a decrease in signal intensity and broadened transitions (Figure 3.3.9 panel B).
Figure 3.3.8. Low-temperature Mn(II) EPR spectroscopy of CP-Ser and mutant proteins at pH 7.5 (75 mM HEPES, 100 mM NaCl). Left panel: EPR spectra of CP-Ser (A), CP-Ser with 0.6 M NaCl (B), CP-Ser-ΔHis3Asp (C), CP-Ser-ΔHis4 (D) and CP-Ser-ΔΔ (E) in the presence of 0.3 equivalents of Mn(II). A radical signal (S = ½) was masked in E. The protein concentration was approximately 100 μM for each sample. The spectra obtained for CP-Ser-ΔHis4 and CP-Ser-ΔΔ are scaled 4x. Right panel: EPR spectra of CP-Ser (A) and the single point mutants CP-Ser(H17A) (B), CP-Ser(H27A) (C), CP-Ser(H91A) (D), and CP-Ser(H95A) (E) each in the presence of 0.3 equivalents of Mn(II). The CP-Ser spectrum was recorded on a 100 μM sample incubated with 1 mM Ca(II) prior to addition of 20 μM Mn(II). Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.

Mn(II) was titrated into samples of CP-Ser in the absence and presence of Ca(II), and select spectra from these titrations are shown in Figure 3.3.9. Side-by-side comparison of these spectra establishes that there is an increase in the sharpness of the inner resonance in the presence of Ca(II). These inner resonances arise from semi-forbidden \( \Delta m_s = \pm 1, \Delta m_l = \pm 1 \) transitions. In the absence of Ca(II), the signals broaden with increasing amounts of Mn(II), which may be due to the metal population additional and less intense species at higher Mn(II) equivalents. We attribute this behavior to free or weakly associated Mn(II) in the sample. In contrast, titration of Mn(II) into CP-Ser in the presence of
Ca(II) resulted in a step-wise increase in the signal intensity, corresponded to a single high-affinity Mn(II) coordination environment in the presence of Ca(II).

The X-band EPR signal of 110 μM CP-Ser with 60 μM Mn(II) and 1 mM Ca(II) could be simulated with zero-field splittings of $D = 270$ MHz and $E = 81$ MHz (Figure 3.3.9 and Table 3.3.3). These zero-field parameters are small compared to other examples mononuclear Mn(II), and the low $D$ value indicates that CP coordinates Mn(II) in an octahedral ligand environment with small rhombic distortion. Based on our experiments with mutant proteins presented here, we contend that the Mn(II) coordination sphere consists of (A8)His17, (A8)His27, (A9)His91, (A9)His95 and two as-yet unidentified ligands. These additional ligands may derive from solvent water, other buffer components, or amino acid side chains. In particular, the sequence of S100A9 contains an extended C-terminal tail region that is disordered in the crystal structure of Ca(II)-bound CP and contains multiple residues with imidazole or carboxylate moieties. The role of the C-terminal tail of S100A9 to high-affinity Mn(II) chelation by CP is discussed in Chapter 4 and Appendix 4.
Figure 3.3.9. Low-temperature EPR titrations of CP-Ser with Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl) in the absence (left panel) and presence (right panel) of 1.0 mM Ca(II). Left panel: Titration of 100 µM CP-Ser with 0.1 (A), 0.5 (B), and 1.0 (C) equivalents of Mn(II). Right panel: Titration of 115 µM CP-Ser with 0.1 (A), 0.5 (B) and 1.0 (C) equivalents of Mn(II) in the presence of Ca(II). Simulation (D) of X-band (9.3 GHz) EPR spectrum (B, green) of 110 µM CP-Ser with 60 µM Mn(II) in the presence of 1.0 mM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Simulation parameters: $S = 5/2$, $I = 5/2$, $a = 8.9$ mT, $g = 2$, $D = 0.009$ cm$^{-1}$, $E/D = 0.30$. The simulation could be fit with no distribution in the zero-field ($D$, $E/D$) parameters and was quantitative for the amount of Mn(II) added. Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.

Table 3.3.3. Reported Zero-Field Splitting Parameters for Mononuclear Mn(II) Determined by EPR.$^a$

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<th>$E$ (MHz)</th>
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$^a$Additional zero-field splitting parameters are given in ref. 24.
3.3.5 CP Prefers to Coordinate Zn(II) at the His₄ Site

Current work demonstrates the CP is able to coordinate Zn(II) at site 1 and site 2 with high affinity, and only site 2 coordinates Mn(II) with high affinity. A comparison of the Zn(II) and Mn(II) $K_d$ values indicates that CP has a thermodynamic preference for Zn(II) coordination at site 2, in agreement with the Irving-Williams series.²⁶ We hypothesize that Zn(II) will displace Mn(II) from the His₄ site, and we monitored Mn(II) release from CP-Ser-ΔHis₃Asp following addition of Zn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Protein precipitation occurred when Ca(II) was included in the mixture, so this experiment was performed in the absence of Ca(II). Following the addition of Zn(II) to the Mn(II)-CP complex, a gradual reduction in the signal intensity was observed over the course of 1 h. (Figure 3.3.10) The intensity of the first allowed transition decreased, and a new feature appeared at $g = 2.17$. These observations are consistent with displacement of Mn(II) from the His₄ site in the presence of Zn(II), and growth of free Mn(II).

![Figure 3.3.10](image_url)

**Figure 3.3.10.** Time course displacement of Mn(II) from the His₄ site following Zn(II) addition at pH 7.5 (75 mM HEPES, 100 mM NaCl) monitored by low-temperature EPR spectroscopy. (A) 120 μM CP-Ser-ΔHis₃Asp with 0.33 equivalents of Mn(II). (B) One equivalent of Zn(II) was added to the sample in A, and immediately frozen with liquid nitrogen and the EPR signal was recorded. This sample was then thawed and allowed to incubate at room temperature for 30 min (C) and 60 min (D). All spectra are scaled for the concentration of added Mn(II). Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.
3.4 Summary and Perspectives

The work presented in this chapter establishes that (i) CP coordinates one equivalent of Mn(II) with high affinity, (ii) the unusual interfacial His$_4$ motif is responsible for Mn(II) coordination, (iii) Ca(II) ions modulate the affinity of CP for Mn(II), and (iv) CP site 2 provides an octahedral coordination sphere for Mn(II). Optical absorption experiments presented in Chapter 2 establish that CP site 2 also coordinates Co(II) in an octahedral ligand field. We have established that (A8)His17, (A8)His27, (A9)His20, and (A9)Asp30 contribute to the Mn(II) coordination sphere, and two ligands remain unidentified. These additional ligands may be solvent water, another buffer component (e.g. chloride), or derived from the protein itself. These ligands are identified in Chapter 4. His$_4$ motifs are rare in biological systems, and a search of the PDB yielded only a few other examples. Two structurally characterized proteins that utilize tetrahistidine motifs to coordinate Mn(II) are the photochemical reaction center from *Rhodobacter sphaeroides* and a cupin from *Thermotoga maritima*. These systems have not been studied by spectroscopic techniques, and to the best of our knowledge this work provides the first EPR characterization of a biological His$_4$ Mn(II)-binding site.

The nanomolar $K_d$ value for site 2 is remarkably high-affinity in the context of other Mn(II)-binding proteins including the metalloregulatory proteins *Bacillus subtilis* MntR ($K_d = 160$ µM) and *Bacillus anthracis* AntR ($K_{d1} = 210$ µM, $K_{d2} = 17$ µM). However, reported affinities for Mn(II) solute-binding proteins for *Streptococcus pneumoniae* PsaA ($K_d = 3.3$ nM) and *Staphylococcus aureus* MntC ($K_d = 4$ nM) are in the low nanomolar range. We propose the CP tetramer may compete with these transporters for Mn(II); however, additional studies will be required to ascertain the metals sequestered by CP *in vitro* and in the context of infection.
Acknowledgements

The low-temperature EPR measurements, ZP1 titrations, and room-temperature $K_d$ determination experiments and analysis were performed by Dr. Joshua A. Hayden, and Ms. Aleth Gaillard performed the Mn(II) thermal denaturation experiments. The CP-Ser(H27D) mutant was prepared and characterized by Ms. Lisa Cunden. Financial support for this work was provided by the Searle Scholars Program (Kinship Foundation), the MIT Center for Environmental Health Sciences (NIH P30-ES002109), and the Department of Chemistry at MIT. We thank Professor Stephen J. Lippard for use of his atomic absorption spectrometer, Dr. Justin J. Wilson for assistance with the AA measurements, Dr. Jeff Simpson for assistance with the EPR spectrometer, Ms. Sumin Kim for assistance with protein purification, and Dr. Andrew J. Wommack for synthesizing ZP1. We thank Professor Michael Hendrich for helpful discussions about the EPR spectroscopic data, and for generously providing the SpinCount simulation software. EPR instrumentation is housed in the Department of Chemistry Instrumentation Facility. Instrumentation for circular dichroism spectroscopy is provided by the MIT Biophysical Instrumentation Facility for the Study of Complex Macromolecular Systems, which is supported by grants NSF-0070319 and NIH GM68762.
References


(2) Kehl-Fie, T. E.; Chitayat, S.; Hood, M. I.; Damo, S.; Restrepo, N.; Garcia, C.; Munro, K. A.; Chazin, W. J.; Skaar, E. P. Cell Host Microbe 2011, 10, 158.


5522.


Chapter 4: Contributions of the C-terminal Tail of S100A9 to High-Affinity Manganese(II) Binding by Human Calprotectin

This chapter was adapted from \textit{J. Am. Chem. Soc.} \textbf{2013}, \textit{135}, 17804-17817.
4.1 Introduction

Calprotectin (CP) comprises ca. 40% of the cytosolic protein in human neutrophils\(^1\) and plays a unique role in Mn(II) sequestration by the innate immune system.\(^2\) As described in Chapter 3, we discovered that CP utilizes the interfacial His\(_4\) motif (site 2) for high-affinity Mn(II) coordination. Furthermore, the Mn(II)-CP EPR signal observed is consistent with six-coordinate Mn(II) in an octahedral coordination sphere, indicating that two as-yet-unidentified ligands contribute to Mn(II) sequestration.\(^4\) These additional ligands may derive from solvent water or hydroxide, additional buffer components such as chloride, or additional protein sidechains. In particular, the S100A9 polypeptide sequence reveals an extended C-terminal extension that is disordered in the crystal structure of Ca(II)-bound CP (Figure 4.1.1). Our prior studies indicated that the S100A9 C-terminal tail does not contribute to the formation of an additional Zn(II)-binding site; however, we hypothesized that this region may complete the six-coordinate motif at site 2. Moreover, the affinity of CP for Mn(II) is very high relative to other Mn(II)-binding proteins, and we reasoned that the C-terminal tail may protect this kinetically labile metal ion from solvent. This C-terminal tail contains several residues that may contribute to transition-metal-ion coordination, namely Glu96, Asp98, Glu99, His103, His104, His105, and Glu111. During the course of our investigations into the C-terminal tail, a crystal structure of Mn(II)- and Ca(II)-bound CP was published.\(^5\) This structure confirmed that the \(\alpha_2\beta_2\) tetramer coordinated Mn(II) at site 2 in a His\(_8\) motif with contributions from (A8)His17, (A8)His27, (A9)His91, (A9)His95, (A9)His103, and (A9)His105.\(^5\) This His\(_8\) coordination motif is unprecedented among known biological Mn(II) coordination motifs (Table 4.1.1).
Figure 4.1.1. Structural features of human CP and proposed mechanism of antimicrobial activity. (A) Model of the CP αβ dimer, taken from the structure of the Ca(II)-bound tetramer. S100A8 is shown in green, and S100A9 is shown in blue. Ca(II) ions are colored as yellow spheres, and transition metal-ion binding residues are shown as orange sticks. PDB: 1XK4. (B) Proposed mechanism of antimicrobial action. CP is stored in cells as the apo αβ dimer. CP is released at sites of infection, where it undergoes further oligomerization to the α2β2 tetramer and becomes a potent chelator, which competes with pathogenic metal-ion transporters and enzymes for bioavailable Mn(II) and Zn(II). (C) Site 2, the His4 motif, is formed at the dimer interface by (A8)His17, (A8)His27, (A9)His91, and (A9)His95. PDB: 1XK4. (D) The Mn(II)-His9 site, the Mn(II) coordination sphere is completed by (A9)His103 and (A9)His105. The Mn(II) ion is shown as a magenta sphere. PDB: 4GGF. (E) Site 1, the His3 Asp motif, is formed at the dimer interface by (A8)His83, (A8)His87, (A9)His20, and (A9)Asp30. PDB: 1XK4. (F) The Mn(II)-His3 Asp site of human CP. The Mn(II) ion, shown as a magenta sphere, was refined with an occupancy of 0.5. PDB: 4GGF. (G) Sequence of alignment of S100A8 and S100A9 with S100A7, S100A12, and S100B. The secondary structural elements of S100A8 and S100A9 are color-coded and shown above the alignment. Cysteine residues mutated to serine for metal-binding studies are colored red, and transition metal-binding residues are colored orange. The C-terminal tail of S100A9 is underlined.
This chapter describes the design and characterization of a family of CP mutants designed to evaluate the contribution of the C-terminal tail of S100A9 (residues 96-114) to high-affinity Mn(II) coordination by CP in solution. We characterize single-point mutants of the acidic residues in the S100A9 C-terminal tail as well as His103, His104, and His105. These studies provide critical new insights into site 2 and include evaluation of flexibility in the C-terminal tail.
4.2 Experimental

4.2.1 Materials and General Methods

All solvents, reagents, and chemicals were obtained from commercial suppliers and used as received unless noted otherwise. Experimental details for the preparation of buffers for metal-binding studies are reported elsewhere. All aqueous solutions were prepared with Milli-Q water (18.2 MΩ cm, 0.22 μm filter). For metal-binding experiments, HEPES buffer was prepared with metal-free Ultrol grade HEPES (free acid, Calbiochem) and TraceSELECT NaCl (Sigma), and metal-free aqueous NaOH (Sigma) was used to adjust the pH. To reduce metal-ion contamination, Teflon-coated spatulas were used to transfer buffer reagents, and buffers were treated with Chelex 100 resin (Biorad, 10 g/L) by stirring in a polypropylene beaker for at least 1 h before use. The Chelex resin was removed by centrifugation or by passing the buffer through a 0.22-μm filter, and all buffers were stored in polypropylene containers. All metal-binding studies were conducted at pH 7.5 in 75 mM HEPES, 100 mM NaCl unless specified otherwise. A Tris buffer (1 mM Tris, pH 7.5) prepared from Tris base (J. T. Baker) was used for circular dichroism (CD) spectroscopy experiments requiring Mn(II) addition. This buffer was treated with Chelex resin for 1 h (10 g/L), filtered through a 0.22-μm filter, and the pH was readjusted to 7.5 with hydrochloric acid. Calcium chloride and 99.999% manganese chloride were purchased from Alfa Aesar. Stock solutions of Ca(II) (1 M) and Mn(II) (1 M) were prepared by using Milli-Q water and acid-washed volumetric glassware, and the solutions were immediately transferred to and stored in polypropylene containers. Working M(II) stocks were prepared daily by diluting the appropriate stock to the desired working concentration with buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). Zinpyr-1 (ZP1) was synthesized from 2’,7’-dichlorofluorescein and di(2-picolyl)amine as described elsewhere. Stock solutions of ZP1 (1.8 mM) were prepared in
anhydrous DMSO, partitioned into 50-μL aliquots, and stored at -20 °C. Each aliquot of ZP1 was freeze-thawed only once.

4.2.2 Electrospray Ionization Mass Spectrometry

Details for ESI-MS are provided in Chapter 2.

4.2.3 Analytical Size Exclusion Chromatography

An ÄKTA purifier (GE Lifesciences) outfitted with a 500-μL sample loop and a Superdex S75 10/300 GL column was used to perform all analytical size exclusion chromatography (SEC) experiments (see Chapter 2 for details). In a typical SEC experiment, the protein was buffer-exchanged from the storage buffer into the running buffer if necessary. The protein concentration was adjusted to the indicated concentration, and Ca(II) or Mn(II) was added for the metal-containing samples. For the Ca(II) titrations, the running buffer was 20 mM HEPES, 100 mM NaCl at pH 8.0. For all other experiments, the running buffer was 75 mM HEPES, 100 mM NaCl at pH 7.5. Following addition of Mn(II), the samples were incubated for 0, 2 or 18 h at 4 °C. Each sample (100 or 200 μL) was loaded onto a 500-μL loop. The column was equilibrated with 1.5 column volumes of running buffer prior to each run. The loop was emptied with 0.5 mL of running buffer, and the protein was eluted over one column volume at a flow rate of 0.5 mL/min at room temperature. The molecular weights were determined from the elution volume by using the linear relationship provided by the calibration curve.

4.2.4 Circular Dichroism Spectroscopy

An Aviv Model 202 circular dichroism (CD) spectrometer maintained at 25 °C was used to collect CD spectra. A 1-mm path-length quartz CD cell (Hellma) was employed for all CD measurements. Protein solutions (10 μM, 300 μL) were prepared immediately prior to data acquisition. CD spectra of the CP mutants in the presence and absence of Ca(II) were
obtained for samples prepared at pH 8.5 (1 mM Tris, 0.5 mM EDTA) for comparison with literature spectra.\textsuperscript{4,15} A 6-μL Ca(II) aliquot was added from a freshly-prepared 100 mM solution in buffer to provide a final concentration of 2 mM, and spectra were recorded ca. 15 min after addition. The CD spectra were recorded from 195 nm to 260 nm at 1-nm intervals. Each data point was averaged for 3 sec before being recorded. Three independent scans were performed for each sample, and the resulting data were averaged to obtain the reported CD spectra. Thermal denaturation experiments were conducted with CP mutant samples prepared in the absence and presence of excess Ca(II) as described above. Manganese-containing samples were prepared at pH 7.5 (1 mM Tris, Chelex-treated). Thermal denaturation experiments were conducted for Mn(II)-CP samples prepared in the absence and presence of Ca(II). A 6-μL aliquot of a 100 mM Ca(II) stock solution (1 mM Tris, pH 7.5, Chelex-treated) was added to the protein to provide a final Ca(II) concentration of 2 mM. Next, a 3-μL Mn(II) aliquot from a 10 mM stock solution in buffer was added immediately before initiating the data collection. The final Mn(II) concentration was 100 μM (10 equivalents / CP). For the thermal denaturation experiments, the CD signal at 222 nm was recorded (3 sec averaging time). The temperature was raised from 25 to 95 °C in increments of 2 °C, and the sample was allowed to equilibrate for one minute prior to data collection.

4.2.5 Mn(II) Competition Experiments with ZP1

For competition experiments between ZP1 and the protein of interest (CP-Ser or mutant), a 2-mL solution of ZP1 (1 μM) was prepared in a quartz cuvette (75 mM HEPES, 100 mM NaCl, pH 7.5) and was allowed to equilibrate in the dark for one hour. The initial emission spectrum was recorded, and the protein was added to the solution from a freshly-thawed aliquot for a final concentration of 4 μM. The solution was allowed to equilibrate in the dark for another 20 min, and the emission spectrum was recorded again. The emission
spectrum of apo ZP1 is not affected by the presence of CP/mutant $\alpha\beta$ or $\alpha_2\beta_2$.\textsuperscript{17} Hence, if the intensity of the emission spectrum varied from the initial spectrum indicating a potential metal-ion contamination, the solution was discarded. In experiments requiring calcium, Ca(II) was then added from a 100 mM working solution (75 mM HEPES, 100 mM NaCl, pH 7.5) to afford a final concentration of 200 $\mu$M (50 equivalents / CP). After Ca(II) addition, the solution was incubated in the dark for 10 min, and the emission spectrum was recorded. A working solution of Mn(II) (500 $\mu$M) was prepared daily at pH 7.5 (75 mM HEPES, 100 mM NaCl). The competition experiments were conducted by titrating Mn(II) into the mixture and allowing the solution to equilibrate for 10 min in the dark after each addition prior to recording the emission spectrum. The emission spectra were recorded from 500 to 650 nm ($\lambda_{ex} = 490$ nm, 0.4 mm excitation and emission slit widths, 10 nm/sec scan rate) and integrated over this range. The integrated emission was normalized to that of the Mn(II)-free sample. Each titration was repeated in triplicate and the resulting averages are reported.

4.2.6 Electron Paramagnetic Resonance Spectroscopy

Electron paramagnetic resonance (EPR) spectra (X-band, 9 GHz) were recorded on a Bruker EMX spectrometer outfitted with an ER 4199HS cavity. A flat quartz cell (Bruker Biospin), positioned in an E-field null plane within the cavity, was used for all room-temperature measurements. An ESR900 cryostat was employed for all low-temperature measurements, and the temperature was measured using a Cernox sensor. A copper-EDTA spin standard was used to account for all relevant intensity factors at low temperature. Low-temperature EPR samples were housed in 4-mm (OD) quartz EPR tubes and frozen in liquid nitrogen prior to spectral acquisition. All spectra were analyzed with the SpinCount software package developed by Michael P. Hendrich at Carnegie Mellon University.
4.2.7 Room-Temperature EPR Spectroscopy

Each titration was performed on a 300-μL scale with 100 μM CP. Working Mn(II) stocks were quantified by comparing the intensities of the inner four transitions of the free Mn(II) signal to that of an atomic absorption standard (J. T. Baker). Mn(II) was added to the protein solution from a working stock solution, and the sample was incubated for 5 min following each Mn(II) addition. All spectra were recorded with a power of 2 mW. The concentration of free Mn(II) was determined by comparing the intensities of the inner four transitions to those of an atomic absorption standard. For titrations performed with CP-Ser-AAA and CP-Ser-Δ101, only the peak intensities varied with each Mn(II) addition.

4.2.8 Site-Directed Mutagenesis

A modified Quick-Change site-directed mutagenesis protocol was employed to generate the mutants (Tables 4.2.1 and 4.2.2). PCR amplification was conducted using PfuTurbo DNA polymerase. For the AHA(K106H), AAE, and AAA(L109H)(E111H) mutant plasmids, the PCR mix contained 5% DMSO. For the AAA(K106H) mutant plasmid, the PCR mix contained 10% DMSO. The annealing temperature of the PCR protocol for each mutant was modified based on the melting temperature of the primers determined by OligoAnalyzer 3.1 (Integrated DNA Technologies). For the AHA(K106H) mutant, the PCR protocol was: 95 °C for 30 sec; 95 °C for 30 sec, 64 °C for 1 min, 68 °C for 17 min (25x); and 4 °C hold. For the AAA(K106H) and AAE mutants, the PCR protocol was: 95 °C for 30 sec; 95 °C for 30 sec, 63 °C for 1 min, 68 °C for 17 min (25x); and 4 °C hold. For the AAA(L109H)(E111H) mutant, the PCR protocol was: 95 °C for 30 sec; 95 °C for 30 sec, 64 °C for 1 min, 68 °C for 17 min (25x); and 4 °C hold. Following PCR amplification, the template plasmid was digested with DpnI (New England Biolabs) by adding two 1-μL aliquots of the restriction enzyme to a 25-μL PCR reaction at t = 0 and 1.5 h and incubating for 3 h at 37 °C. The digestion products were transformed into chemically-competent *E. coli* TOP10 cells.
Overnight cultures (5 mL, 50 µg/mL kanamycin) were grown from single colonies, and the purified plasmids were isolated by using a miniprep kit (Qiagen). The DNA sequences and the presence of the desired mutations were verified by DNA sequencing (MIT Biopolymers).

Table 4.2.1. Primers Employed for Site-Directed Mutagenesis of the S100A9 C-Terminal Tail.\(^a\)

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\(^a\) Bold codons indicate stop codons. The codons containing mutations are underlined and colored red.
<table>
<thead>
<tr>
<th>Template</th>
<th>Product</th>
<th>Primer Pairing</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET41a-S100A9(C3S)</td>
<td>pET41a-S100A9(C3S)(G102Stop)</td>
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<tr>
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<td></td>
<td>G102Stop-2</td>
</tr>
<tr>
<td>pET41a-S100A9(C3S)</td>
<td>pET41a-S100A9(C3S)(E96A)</td>
<td>E96A-1, E96A-2</td>
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<tr>
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<td>pET41a-S100A9(C3S)(D98A)</td>
<td>D98A-1, D98A-2</td>
</tr>
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<td>pET41a-S100A9(C3S)(E99A)</td>
<td>E99A-1, E99A-2</td>
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<td>(H103A)(H105A)(K106H)</td>
<td>AHA(K106H)-2</td>
</tr>
<tr>
<td>pET41a-S100A9(C3S)</td>
<td>pET41a-S100A9(C3S)</td>
<td>AAA(K106H)-1,</td>
</tr>
<tr>
<td>(H103A)(H104A)(H105A)</td>
<td>(H103A)(H104A)(H105A)(K106H)</td>
<td>AAA(K106H)-2</td>
</tr>
<tr>
<td>pET41a-S100A9(C3S)</td>
<td>pET41a-S100A9(C3S)</td>
<td>AAE-1, AAE-2</td>
</tr>
<tr>
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<td>(H103A)(H104A)(H105E)</td>
<td></td>
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<tr>
<td>pET41a-S100A9(C3S)</td>
<td>pET41a-S100A9(C3S)</td>
<td>(L109H)(E111H)-1,</td>
</tr>
<tr>
<td>(H103A)(H104A)(H105A)</td>
<td>(H103A)(H104A)(H105A)</td>
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</tr>
<tr>
<td></td>
<td>(L109H)(E111H)</td>
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</tr>
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</table>

**4.2.9 Preparation of Mutant CP**

Mutants of human calprotectin were purified as described previously. Protein yields ranged from ca. 4 mg (CP-Ser-Δ101) to ca. 40 mg (others). The Δ101 mutant was purified multiple times over the course of these investigations, and other CP-Ser mutants were overexpressed and purified once. The chimeric protein CP-Ser-mT, which consists of human A9 residues 1-95 followed by mouse A9 residues 97-113, was lysed in BME-containing buffer and dialyzed into 20 mM HEPES, pH 8.0 without a reducing agent. The purified protein was found to contain a BME-adduct of S100A9 resulting from disulfide bond formation between BME and a cysteine at position 110 (mT chimera numbering). Prior to use in select metal-binding studies, CP-Ser-mT was reduced with TCEP and buffer-exchanged into a buffer that had been purged with nitrogen or argon. Briefly, a 50-μL aliquot of CP-Ser-mT (ca. 600 μM) was thawed on ice and diluted to 500 μL with metal-binding
buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). TCEP was added to a final concentration of 1 mM, and the protein was incubated on ice for 30 min. The protein was buffer exchanged into nitrogen- or argon-purged buffer, and the identity of reduced CP-Ser-mT was verified by ESI-MS (Table 4.3.2).

The synthetic genes for S100A9(C3S)(H103A)(H104A)(H105A), S100A9(C3S)(K106A), S100A9(C3S)(E111A), and S100A9(C3S)-mT were acquired from DNA 2.0. The _E. coli_ optimized nucleotide sequences, as well as the translated protein sequences, are provided below.

**Ndel-S100A9(C3S)(H103A)(H104A)(H105A)-Stop-Xhol**

_E. coli_ optimized nucleotide sequence:

CATATGACGAGCAAAATGAGCCAACTGGAACGCAACATCGAGACTATTATCAACACTTT
TCACCAGTACTCTGTCAAACCTGCCATCCGGACACCCTGAATCAGGGTGAGTCTCAA
GAACTGGTGCGTAAAGACCTGCAGAATTTTCTGAAAAAGGAAGAACAAAAACGAGAAGG
TTATCGAGCAGACATTATGGGAAGATCTGGATACCAATGCCGATAAGCAACTGAGCTCGA
GGATTTCATTATGCTGATGGCGCGTTTGACGTGGGCATCCCACGAAAAGATGCATGAGG
GTGACGAAGGTCCGGGTGCGGCTGCGAAGCCAGGCTTGGGTGAGGGCACCCGTAA
CTCGAG

Translated sequence for Ndel-S100A9(C3S)(H103A)(H104A)(H105A)-Stop-Xhol:

HMTSKMSQLERNIEITINTFHQYSVKLGHPDTLNQGEFKELVRK
DLQNFLKKEKNEKVENHEHIMEDLDTNADKQLSFEEFIMLMARLT
WASHKEMHEDGDEPGAAAAKPGGLGEGTStop LE
**Ndel-S100A9(C3S)(K106A)-Stop-Xhol**

*E. coli* optimized nucleotide sequence:

CATATGACGAGCAAAATGAGCCAAACTGGGAACGCAACATCGAGACTATTATCAACACTTT
TCACCAGTACTCTGTCAAACACTGGGCCATCCGGACACCCTGAATCAGGGTGAGTTCAAA
GAACTGGGTGCGTAAAGACCTGCGAAGATATTCTGAAAAAGGAGAACAACACGAGAAGG
TTATCGAGGCACATTAGGAAGATCTGGATACCAATCGCATAAGCAACTGAGCTTCGAA
GAGTTCTATTATGCATGCGGCGCGTTTGACGTGGGCATCCCAGAAAAGATGCATGAGG
GTGACGAAGGCTCCGGGTCAACCATACGCGCCAGGCTTGGGTAGGTCAGGGCATCACCCTACG

Translated sequence for Ndel-S100A9(C3S)(K106A)-Stop-Xhol:

HMTSKMSQLERNIETTINTFHQYSVKLGHPDNLQGEFKELVRK
DLQNFLLKKENKNEKVIEHIMEDLDTNADKQLSFEFIMLMARLT
WASHHEKMHEGDEGPQGHHAPGLGEOTP Stop LE

**Ndel-S100A9(C3S)(E111A)-Stop-Xhol**

*E. coli* optimized nucleotide sequence:

CATATGACGAGCAAAATGAGCCAAACTGGGAACGCAACATCGAGACTATTATCAACACTTT
TCACCAGTACTCTGTCAAACACTGGGCCATCCGGACACCCTGAATCAGGGTGAGTTCAAA
GAACTGGGTGCGTAAAGACCTGCGAAGATATTCTGAAAAAGGAGAACAACACGAGAAGG
TTATCGAGGCACATTAGGAAGATCTGGATACCAATCGCATAAGCAACTGAGCTTCGAA
GAGTTCTATTATGCATGCGGCGCGTTTGACGTGGGCATCCCAGAAAAGATGCATGAGG
GTGACGAAGGCTCCGGGTCAACCATACGCGCCAGGCTTGGGTAGGTCAGGGCATCACCCTACG
TTCGAG
Translated sequence for Ndel-S100A9(C3S)(E111A)-Stop-Xhol:

W A S H E K M H E G D E G P G H H H K P G L G A G T P Stop L E

Design of the Synthetic gene for S100A9(C3S)-mT

S100A9(C3S)-mT is an 112-aa chimera of the human and mouse S100A9 amino acid sequences. The N-terminal 95 residues correspond to residues 1-95 of human S100A9(C3S). The C-terminal 17 residues correspond to residues 97-113 of mouse S100A9. The mouse portion is indicated by the italics font below.

A synthetic gene for human S100A9(C3S)-mT was ordered from DNA 2.0 and optimized for E. coli codon usage. The synthetic gene was designed to include a N-terminal Ndel restriction site (N-terminal Met residue encoded by the Ndel site) and a C-terminal stop codon followed by a Xhol restriction site. This synthetic gene was ligated into the Ndel and Xhol restriction sites of pET-41a by DNA 2.0.

Ndel-S100A9(C3S)-mT-Stop-Xhol

E. coli optimized nucleotide sequence:

CATATGACGAGCAAATGAGCCCAACTGGAACGTAACATCGGAACATATTATCAACACTTT
TCACCAGTACAGCGTCAAGCTGGCCATCCGGACACCTTGAACCAGGGTGAGTTCAAA
GAGCTGGTGCAGCAGATCTGGGAGCCACACCTTGAACCAGGGTGAGTTCAAA
TTATTGAACACATTATGGAAGATCTGGACACCAATGCAGACAAACAACTGTCTTTTGAA
GAGTTCATCATGCTGATGGCCCGTTTGACCTGGGCGAGCCACGAGAAGATGCATGA
ACAATCCGCGTCATGCTGCTACTCCACGCTAAGGGTTGCGGCAATTAACTCGAG


**Translated sequence for Ndel-S100A9(C3S)-mT-Stop-Xhoi:**

```
HM TSKMSQLERNIETIINTFHQYSVKLGHPDTLNQGEFKELVRK
DLQNFLKKENKNEKVIEHIMEDLDTNADKQLSFEEFIMLMARLT
WASHEKMHENMPRGHGSHHKGCNGKStopLE
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**Table 4.2.3. Calprotectin protein nomenclature.**

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<th>MUTATIONS</th>
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<td>CP-Ser ΔΔ</td>
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<td>CP-Ser(E96A)</td>
<td>(C42S)</td>
</tr>
<tr>
<td>CP-Ser(D98A)</td>
<td>(C42S)</td>
</tr>
<tr>
<td>CP-Ser(E99A)</td>
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<tr>
<td>CP-Ser(H103A)</td>
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</tr>
<tr>
<td>CP-Ser(H104A)</td>
<td>(C42S)</td>
</tr>
<tr>
<td>CP-Ser(H105A)</td>
<td>(C42S)</td>
</tr>
<tr>
<td>CP-Ser(K106A)</td>
<td>(C42S)</td>
</tr>
<tr>
<td>CP-Ser(E111A)</td>
<td>(C42S)</td>
</tr>
<tr>
<td>CP-Ser-AHA</td>
<td>(C42S)</td>
</tr>
<tr>
<td>CP-Ser-AAA</td>
<td>(C42S)</td>
</tr>
<tr>
<td>CP-Ser-AAE</td>
<td>(C42S)</td>
</tr>
<tr>
<td>CP-Ser-AAA(K106H)</td>
<td>(C42S)</td>
</tr>
<tr>
<td>CP-Ser-AHA(K106H)</td>
<td>(C42S)</td>
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<tr>
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<td>(C42S)</td>
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<tr>
<td>CP-Ser-mT</td>
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<td>Molecular Weight ($\text{Da}^a$)</td>
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<td>S100A9(C3S)(D98A)</td>
<td>13,181.8</td>
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<td>S100A9(C3S)(E99A)</td>
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<tr>
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<td>S100A9(C3S)(mT)</td>
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$^a$ Molecular weights were calculated by using the ProtParam tool available on the ExPASy server (http://web.expasy.org/protparam).
$^b$ Extinction coefficients (280 nm) were calculated by using the ProtParam tool.
4.3. Results and Discussion

4.3.1. Design and Preparation of the Human S100A9 Mutant Family

The C-terminal region of human S100A9 contains four acid residues (Glu96, Asp98, Glu99, and Glu111) and three histidine residues (His103, His104, and His105). We designed a 16-member mutant family to evaluate the contribution of each of these residues to high-affinity Mn(II) coordination at site 2, which is characterized by an interfacial His₄ motif (Table 4.3.1). These mutant proteins are based on CP-Ser, a heterooligomer of S100A8(C42S) and S100A9(C3S) that we have previously employed in antibacterial assays and metal-binding studies.⁴ The K106A mutant was made to study the effect of mutating a non-coordinating amino acid in the region of the Mn(II) binding site. Additional tail mutants were designed to evaluate the flexibility of the glycine-rich S100A9 C-terminal tail. CP-Ser-AAA(K106H), CP-Ser-AHA(K106H), and CP-Ser-AAA(L109H)(E111H) were designed to evaluate the importance of His positioning. CP-Ser-AAE was prepared to probe the consequences of a N→Oₓ (x = 1,2) donor substitution on Mn(II) complexation. CP-Ser-Δ101 is a truncation of S100A9 that lacks residues 102-114 of the C-terminal region. Lastly, CP-Ser-mT is a chimera of human and mouse S100A9 were the C-terminal 19 residues of S100A9 are substituted by the C-terminal 17 residues of the murine homologue. The murine tail region houses two HXH motifs at positions 102-104 and 104-106, and a single Cys residue at position 110. Two CP mutant proteins with modifications to the S100A9 C-terminus were recently reported and are analogous to the CP-Ser-Δ101 and CP-Ser-AAA mutants presented in this chapter.

Each mutant protein was purified as described previously for CP-Ser,¹⁵ and they were characterized by SDS-PAGE, mass spectrometry (Table 4.3.2), analytical SEC (Appendix 2 and Table 4.3.3), and CD spectroscopy (Appendix 2). Each protein was obtained in high purity, had the expected α-helical fold, and exhibited Ca(II)-dependent
tetramerization. CP-Ser-mT was purified in the presence of BME to prevent cysteine oxidation, and ESI-MS established that CP-Ser-mT was purified as a BME adduct (CP-Ser-mT-BME). A TCEP-reduction protocol was employed to obtain CP-Ser-mT with a free cysteine residue. Following reduction, CP-Ser-mT was handled aerobically and displayed no signs of precipitation.

Table 4.3.1. Amino Acid Sequences of the C-terminal Tail of CP-Ser and Mutants.\textsuperscript{a}

<table>
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<th>Protein\textsuperscript{b}</th>
<th>Sequence\textsuperscript{c,d,e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-Ser</td>
<td>\textsuperscript{a9}(1-90)-HEKMHEGDEG PGHHHKPGLG EGTP</td>
</tr>
<tr>
<td>CP-Ser-Δ101</td>
<td>\textsuperscript{a9}(1-90)-HEKMHEGDEG P</td>
</tr>
<tr>
<td>CP-Ser-mT</td>
<td>\textsuperscript{a9}(1-90)-HEKMHEGNPR \textsuperscript{G}GHGSHGKG\textsuperscript{G}C \textsuperscript{G}K</td>
</tr>
<tr>
<td>CP-Ser(E96A)</td>
<td>\textsuperscript{a9}(1-90)-HEKMHA\textsuperscript{G}DEG PGHHHKPGLG EGTP</td>
</tr>
<tr>
<td>CP-Ser(D98A)</td>
<td>\textsuperscript{a9}(1-90)-HEKMHEG\textsuperscript{A}EG PGHHHKPGLG EGTP</td>
</tr>
<tr>
<td>CP-Ser(E99A)</td>
<td>\textsuperscript{a9}(1-90)-HEKMHEG\textsuperscript{D}AG PGHHHKPGLG EGTP</td>
</tr>
<tr>
<td>CP-Ser(H103A)</td>
<td>\textsuperscript{a9}(1-90)-HEKMHEGDEG PG\textsuperscript{A}HHKPGLG EGTP</td>
</tr>
<tr>
<td>CP-Ser(H104A)</td>
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<td>CP-Ser(K106A)</td>
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</tr>
<tr>
<td>CP-Ser(E111A)</td>
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<tr>
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<td>CP-Ser-AAE</td>
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</tr>
<tr>
<td>CP-Ser-AAA(K106H)</td>
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<tr>
<td>CP-Ser-AHA(K106H)</td>
<td>\textsuperscript{a9}(1-90)-HEKMHEGDEG PG\textsuperscript{A}AHPG\textsuperscript{L}G EGTP</td>
</tr>
<tr>
<td>CP-Ser-AAA(L109H)(E111H)</td>
<td>\textsuperscript{a9}(1-90)-HEKMHEGDEG PG\textsuperscript{A}AKPG\textsuperscript{H}GHG\textsuperscript{H}G EGTP</td>
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</table>

\textsuperscript{a} See Table 4.2.3 for protein nomenclature and Table 4.2.4 for protein molecular weights and extinction coefficients. \textsuperscript{b} The “CP-Ser” is routinely omitted in the text for mutants. \textsuperscript{c} Amino acid residues derived from the sequence of murine S100A9 are italicized. \textsuperscript{d} Mutated amino acids are colored red. \textsuperscript{e} HxH motifs are underlined.
Table 4.3.2. Mass Spectrometric Analysis of Human CP and Mutants.¹

<table>
<thead>
<tr>
<th>Protein</th>
<th>S100A8 Calculated Mass (g/mol)ᵇ</th>
<th>S100A8 Observed Mass (g/mol)ᶜ</th>
<th>S100A9 Calculated Mass +/-¹⁺Met (g/mol)d</th>
<th>S100A9 Observed Mass (g/mol)²</th>
</tr>
</thead>
<tbody>
<tr>
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<td>10 819.1</td>
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<td>13 182.6</td>
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<td>10 819.0</td>
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<td>10 819.0</td>
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<td>13 160.6</td>
</tr>
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<td>10 819.1</td>
<td>13 159.8</td>
<td>13 160.7</td>
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<td>CP-Ser(K106A)</td>
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<td>10 818.8</td>
<td>13 168.8</td>
<td>13 169.1</td>
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<tr>
<td>CP-Ser(E111A)</td>
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<td>10 818.8</td>
<td>13 089.8</td>
<td>13 166.2</td>
</tr>
<tr>
<td>CP-Ser-mT-BME</td>
<td>10 818.4</td>
<td>10 818.8</td>
<td>13 034.7 (-Met1)</td>
<td>13 035.1</td>
</tr>
</tbody>
</table>

¹ Masses were determined using a denaturing protocol on an Agilent Poroshell 300SB-C18 column over a 0–65% gradient of acetonitrile in 0.1 % formic acid. ² Molecular weights were calculated by using the ProtParam tool available at the ExPasy server (http://web.expasy.org/protparam). ³ Masses were calculated with the Agilent MassHunter BioConfirm software package. ⁴ The N-terminal methionine of S100A9 is sometimes cleaved during overexpression in E. coli. Masses are shown for the full-length and shortened forms of S100A9. ⁵ Mass not found during deconvolution of the raw data. ⁶ Mass of CP-Ser-mT following treatment with TCEP.
Table 4.3.3. Analytical SEC Retention Volumes and Calculated Molecular Weights of CP-Ser and Mutant Proteins in the Absence and Presence of Ca(II).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Elution Volume -Ca(II) (mL)</th>
<th>Calculated Molecular Weight -Ca(II) (kDa)</th>
<th>Elution Volume +Ca(II) (mL)</th>
<th>Calculated Molecular Weight +Ca(II) (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-Ser</td>
<td>11.4</td>
<td>35.5</td>
<td>10.6</td>
<td>49.5</td>
</tr>
<tr>
<td>CP-Ser(E96A)</td>
<td>11.6</td>
<td>32.4</td>
<td>10.8</td>
<td>45.3</td>
</tr>
<tr>
<td>CP-Ser(D98A)</td>
<td>11.6</td>
<td>32.0</td>
<td>10.8</td>
<td>44.9</td>
</tr>
<tr>
<td>CP-Ser(E99A)</td>
<td>11.6</td>
<td>32.1</td>
<td>10.8</td>
<td>45.2</td>
</tr>
<tr>
<td>CP-Ser(H103A)</td>
<td>11.5</td>
<td>33.8</td>
<td>10.7</td>
<td>46.5</td>
</tr>
<tr>
<td>CP-Ser(H104A)</td>
<td>11.5</td>
<td>33.5</td>
<td>10.7</td>
<td>46.3</td>
</tr>
<tr>
<td>CP-Ser(H105A)</td>
<td>11.5</td>
<td>33.7</td>
<td>10.7</td>
<td>46.5</td>
</tr>
<tr>
<td>CP-Ser(K106A)</td>
<td>11.4</td>
<td>34.2</td>
<td>10.6</td>
<td>48.3</td>
</tr>
<tr>
<td>CP-Ser(E111A)</td>
<td>11.5</td>
<td>33.3</td>
<td>10.7</td>
<td>46.7</td>
</tr>
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<td>CP-Ser-AHA</td>
<td>11.5</td>
<td>33.3</td>
<td>10.7</td>
<td>46.7</td>
</tr>
<tr>
<td>CP-Ser-AAA</td>
<td>11.5</td>
<td>34.0</td>
<td>10.6</td>
<td>48.3</td>
</tr>
<tr>
<td>CP-Ser-AAE</td>
<td>11.4</td>
<td>34.8</td>
<td>10.5</td>
<td>49.9</td>
</tr>
<tr>
<td>CP-Ser-AAA(K106H)</td>
<td>11.4</td>
<td>34.2</td>
<td>10.7</td>
<td>47.0</td>
</tr>
<tr>
<td>CP-Ser-AHA(K106H)</td>
<td>11.3</td>
<td>35.6</td>
<td>10.7</td>
<td>47.0</td>
</tr>
<tr>
<td>CP-Ser-AAA(L109H)(E111H)</td>
<td>11.6</td>
<td>31.9</td>
<td>10.9</td>
<td>44.0</td>
</tr>
<tr>
<td>CP-Ser-Δ101</td>
<td>11.8</td>
<td>29.9</td>
<td>11.0</td>
<td>41.6</td>
</tr>
<tr>
<td>CP-Ser-mT</td>
<td>11.7</td>
<td>30.4</td>
<td>10.9</td>
<td>42.9</td>
</tr>
</tbody>
</table>

4.3.2 The C-Terminal Tail Contributes to Ca(II)-Dependent Oligomerization

Analytical SEC reveals that CP-Ser-Δ101 (100 μM) and CP-Ser-AAA (100 μM) completely oligomerize to the heterotetramer when 2 mM Ca(II) is included in the running buffer. We have previously reported step-wise tetramerization of CP-Ser when sub-
stoichiometric equivalents of Ca(II) are included in the protein sample. When CP-Ser was incubated with 8 equivalents of Ca(II) / CP dimer, nearly full conversion to the tetramer was observed. In contrast, with 8 equivalents of Ca(II), the CP-Ser-Δ101 and CP-Ser-AAA mutants exhibit broad elution profiles that roughly correspond to a 1:1 mixture of dimer / tetramer (Figure 4.3.1). These observations indicate that deletion or mutation of the S100A9 C-terminal impacts the ability of CP to tetramerize in the presence of Ca(II). We cannot rule out the possibility that these mutations affect the affinity of the CP EF-hands for Ca(II).

Moreover, this observation is important from the stand point of metal-binding studies because (i) Ca(II) concentrations affect the speciation of CP-Ser and the mutant proteins and (ii) the Mn(II) and Zn(II) affinities of CP are modulated by Ca(II).

**Figure 4.3.1.** Calcium-binding titrations of 100 µL of 100 µM CP-Ser-Δ101 (A) and 100 µM CP-Ser-AAA (B) monitored by SEC at pH 8.0 (20 mM HEPES, 100 mM NaCl). The bold black line is the CP mutant in the absence of Ca(II). The bold red line is a standard for the Ca(II)-bound heterotetramer and corresponds to each mutant eluted with 2 mM Ca(II) in the running buffer. The thin blue, green, purple, and red traces correspond to addition of 1.0, 2.0, 4.0, and 8.0 equiv of Ca(II), respectively, to CP prior to SEC. Absorbance was monitored at 280 nm at room temperature.
4.3.3 Analytical SEC and Thermal Denaturation Studies Support Contribution of the C-Terminal Tail and HHH Motif to Mn(II) Coordination in Solution

We have previously reported that Mn(II) coordination by CP (αβ) at site 2 results in an analytical SEC peak shift to lower elution volume. CP-Ser (αβ) elutes at ca. 11.4 mL (36 kDa), and this peak shifts to ca. 10.6 mL (50 kDa) in the presence of Ca(II). These elution volumes differ from those presented in previous chapters because a different Superdex 75 10/300 GL column was employed in these studies. We reasoned that the SEC elution volumes of the S100A9 C-terminal tail mutants following incubation with Mn(II) may provide insight into whether each mutant coordinated Mn(II). The elution volume behavior may be classified into three groups, and representative data are shown in Figure 4.3.2. Additional data is provided in Appendix 2, Figures A2.49-A2.51. The first group exhibits a Mn(II)-dependent peak shift similar to CP-Ser and consists of CP-Ser(E96A), D98A, D99A, H103A, H104A, H105A, K106A, E111A, and CP-Ser-mT-BME. The Mn(II)-dependent peak has an elution volume that is between the αβ and Ca(II)-induced αβ2 forms. We conclude that these mutants coordinate Mn(II) and retain Mn(II) over the course of the column. The second group consists of CP-Ser-Δ101, CP-Ser-AAA, CP-Ser-AAE, CP-Ser-AAA(K106H), and CP-Ser-AAA(L109H)(E111H). In the presence of Mn(II), these mutants elute as a broad feature that consists of two overlapping peaks. The two-peak behavior may result from a diminished capacity to coordinate and retain Mn(II) over the course of the column. The CP-Ser-AHA and CP-Ser-AHA(K106H) mutants comprise the third group (See Appendix 2, Figures A2.50 and A2.51), and the SEC behavior is characterized by a Mn(II)-induced peak with a shoulder / tail at higher elution volumes. We contend that these mutants coordinate Mn(II), albeit to different degrees. Moreover, the elution of the Mn(II)-dependent peak, which is between the typical dimer and tetramer peaks, indicates a change in protein confirmation. The C-terminal tail region likely wraps in to coordinate Mn(II) in these proteins,
and the ordering of the C-terminal tail and / or Mn(II)-dependent tetramer formation may be responsible for the elution volume of CP with Mn(II). This observation is in general agreement with the crystal structure of Mn(II)- and Ca(II)-bound CP-Ser.\(^5\)

**Figure 4.3.2.** Analytical SEC of CP-Ser and mutant proteins in the absence and presence of 10 equivalents of Mn(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). The proteins were pre-incubated with 10 equivalents of Mn(II) (solid red line). The chromatograms for each protein in the absence (dashed line, \(\alpha\beta\)) and presence (dotted line, \(\alpha_2\beta_2\)) of 2 mM Ca(II) in the SEC running buffer are provided as standards. The protein concentrations were 200 \(\mu\)M with Mn(II) and 100 \(\mu\)M with and without Ca(II). The chromatograms were normalized to maximum peak absorbance values of 1.

We next used CD spectroscopy to ascertain if Mn(II) binding perturbs the \(T_m\) values for the mutant proteins CP-Ser-\(\Delta101\), CP-Ser-AAA, and CP-Ser-AHA (Figure 4.3.3, Table 4.3.4). We have previously reported that Mn(II) coordination stabilizes the secondary structure of the heterodimer and the heterotetramer.\(^4\) CP coordinates Mn(II) at the dimer interface, and we attribute the increase in \(T_m\) to stabilization of the heterooligomers. Addition of Mn(II) to the S100A9 C-terminal tail mutants did increase the stability of these proteins; however, the shift in the \(T_m\) was less than that seen for CP-Ser (Table 4.3.4). The \(T_m\) values for \(\Delta101\) and CP-Ser-AAA increased to 65 °C in the presence of Mn(II), and 88 °C in the
presence of Ca(II) and Mn(II). In contrast, CP-Ser exhibited $T_m$ values of 87 °C and >95 °C in the presence of Mn(II) and Ca(II)/Mn(II), respectively. The mutations of the tail region presented here had no effect on the stability of apo or Ca(II)-bound $\Delta$101 and AAA. This trend supports the notion that CP-Ser-$\Delta$101 and CP-Ser-AAA are able to coordinate Mn(II) under these conditions, and that the C-terminal tail and the HHH motif of S100A9 contribute to Mn(II) complexation by CP in the absence and presence of Ca(II).

![Figure 4.3.3](image)

**Figure 4.3.3.** Thermal denaturation of CP-Ser-$\Delta$101 (A) and CP-Ser-AAA (B) in the absence and presence of 10 equivalents Mn(II) and with or without 2 mM Ca(II) (1 mM Tris-HCl, pH 7.5).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Melting Temperature $-\text{Ca(II)}$ (°C)</th>
<th>Melting Temperature $+\text{Mn(II)}$ (°C)</th>
<th>Melting Temperature $+\text{Ca(II)}$ (°C)</th>
<th>Melting Temperature $+\text{Ca(II)} +\text{Mn(II)}$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-Ser $^b$</td>
<td>61</td>
<td>87</td>
<td>79</td>
<td>&gt;95</td>
</tr>
<tr>
<td>CP-Ser-$\Delta$101</td>
<td>59</td>
<td>65</td>
<td>79</td>
<td>88</td>
</tr>
<tr>
<td>CP-Ser-AAA</td>
<td>61</td>
<td>65</td>
<td>79</td>
<td>88</td>
</tr>
<tr>
<td>CPSer-AHA</td>
<td>n.d. $^c$</td>
<td>67</td>
<td>n.d. $^c$</td>
<td>88</td>
</tr>
</tbody>
</table>

$^a$ The manganese samples contained 10 equivalents of Mn(II). $^b$ Data is taken from Hayden et. al., 2013 $^c$ Not determined
4.3.4. Mn(II)-Binding Titrations by ZP1 Competition and RT-EPR Confirm a Role for the C-Terminal Tail

With support for Mn(II) complexation by the C-terminal tail mutants by analytical SEC, we employed the fluorescent metal sensor ZP1 in a series of Mn(II) competition assays. ZP1 coordinates Mn(II) ($K_{d1} = 550 \text{ nM}$), and this metal-ion quenches fluorescence emission. We performed Mn(II) competition assays in the presence of 50 equivalents Ca(II) / CP dimer, which we expect to promote full conversion of mutant CP to the heterotetrameric form. These results provide a relative measure of the Mn(II) affinities, and the results also facilitate identification of protein-based residues that are required for high-affinity Mn(II) coordination (Figure 4.3.4).

Select competition titrations are shown in Figure 4.3.4 panels A and B, and the full data set is provided in Appendix 2. Direct titration with Mn(II) observed by room-temperature EPR revealed that CP-Ser (Q 2 I1 2) has $K_{d,site2} = 194 \pm 203 \text{ nM}$ for Mn(II) and outcompetes ZP1 for 1 equivalent of Mn(II) / CP-Ser. Several of the mutants compete with ZP1 for Mn(II), and this enhanced competition indicates a lower affinity for Mn(II). The E96A, D98A, E99A, K106A, and E111A mutants exhibit behavior comparable to CP-Ser and outcompete ZP1 for Mn(II). None of the acidic residues of the S100A9 C-terminal tail are essential for high-affinity Mn(II) coordination. CP-Ser-Δ101 and CP-Ser-AAA exhibit nearly identical behavior and afford the greatest competition with ZP1. The titration curves for these mutants are similar to those seen for CP-Ser(H91A), CP-Ser(H95A), and CP-Ser(H27D). These results indicate that deletion of the S100A9 C-terminal tail or mutation of the HHH motif decreases the affinity of CP for Mn(II) but does not completely preclude Mn(II) binding. Moreover, the CP-Ser-AAE competed with ZP1 for Mn(II) and was similar to the CP-Ser-AAA and CP-Ser-Δ101 mutant. This observation indicates that substitution of the HHH motif with Glu does not afford comparable Mn(II) coordination. The titration curves for H103A, H104A, H105A, and AHA mutants fall between the CP-Ser and CP-Ser-AAA mutants and affords a relative
affinity order of CP-Ser ≈ H104A > H103A ≈ H105A > AHA > AAA. The H104 and CP-Ser titration curves are virtually superimposable for up to 0.75 equivalents of Mn(II) added / CP dimer. Movement of the HxH motif from positions 103-105 to positions 104-106 or 109-111 allows ZP1 to compete for Mn(II) and provides a relative affinity ordering of CP-Ser > CP-Ser-AHA(K106H) > CP-Ser-AAA(L109H)(E111H). These ZP1 competition experiments support the importance of the HxH motif to high-affinity Mn(II) binding at site 2. These results may also suggest that the flexibility of the C-terminal tail allows non-native His residues to modulate the affinity of CP for Mn(II) in the absence of the native HxH motif.

The analytical SEC and ZP1 competitions conclusively show that the CP-Ser-AAA and CP-Ser-Δ101 mutants coordinate Mn(II) in the absence and presence of Ca(II), respectively. This result is in disagreement with the conclusions drawn from a recent ITC study in which negligible enthalpy change was observed following titration of the CP-Ser mutants HN (AAA analogue, His→Asn mutations) or ΔTail (Δ101 analogue) with Mn(II). This observation was attributed to no Mn(II) binding under these conditions. To further evaluate the Mn(II)-binding properties of the CP-Ser-AAA and CP-Ser-Δ101 mutants, we employed room-temperature EPR spectroscopy (See Chapter 3, Section 3.3.2). For CP-Ser-Δ101 and CP-Ser-AAA, plots of [Mn(II)\text{free}] / CP vs. [Mn(II)\text{total}] / CP yielded a linear increase in the concentration of free Mn(II) with slopes of 0.7-0.8, indicating an attenuation of the free Mn(II) signal due to Mn(II) coordination by these proteins (Figure 4.3.5, Table 4.3.5). Based on the experiments presented here, we conclude that CP-Ser-Δ101 and CP-Ser-AAA coordinate Mn(II) but with lower affinity than CP-Ser. The competition of these mutants with ZP1 for Mn(II) suggest an affinity in the range of $K_a \approx 10^{-7}$ M. The origins of the discrepancy with the ITC titrations are unclear. ITC directly measures the enthalpic contribution to binding equilibria, and entropy-driven metal-binding events are not detectable by this method. Moreover, the Ca(II) concentration is described as stoichiometric, and the initial speciation of heterodimer / heterotetramer in the ITC experiment is not known.
Figure 4.3.4. Select S100A9 C-terminal tail mutants compete with ZP1 for Mn(II) (75 mM HEPES, 100 mM NaCl, pH 7.0). (A) Δ101 and AAA compete with ZP1 for Mn(II), whereas the D98A and E111A exhibit behavior similar to CP-Ser. (B) Mutants of the HHH sequence compete with ZP1 for Mn(II). In panels A and B, one representative titration for ZP1 only and CP-Ser are shown. (C) Integrated ZP1 emission in the presence of 4 μM CP and 200 μM Ca(II) with 3 (light gray, 0.75 equivalents) or 4 (dark gray, 1.0 equivalent) μM Mn(II). ZP1 is the no-protein control. The data are normalized to apo ZP1 emission. All error bars represent the standard deviation from the mean (n = 3).
Figure 4.3.5. Slope analysis of the room-temperature EPR titrations of CP-Ser-Δ101 (A) and CP-Ser-AAA (B). Plots of \([\text{Mn(II)}_{\text{free}}]\) versus \([\text{Mn(II)}_{\text{total}}]\) are shown. All titrations were performed with 100 µM protein (75 mM HEPES, 100 mM NaCl, pH 7.5). The slope and linear correlation values are given in Table 4.3.5.

Table 4.3.5. Slope Analysis of Room-Temperature EPR Titrations.\(^a\)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Slope</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer(^b)</td>
<td>1.00</td>
<td>0.9967</td>
</tr>
<tr>
<td>CP-Ser-ΔHis(^4)(^b)</td>
<td>1.0452</td>
<td>0.9957</td>
</tr>
<tr>
<td></td>
<td>0.9412</td>
<td>0.9627</td>
</tr>
<tr>
<td>CP-Ser-AAA</td>
<td>0.7738</td>
<td>0.9902</td>
</tr>
<tr>
<td></td>
<td>0.7957</td>
<td>0.9894</td>
</tr>
<tr>
<td>CP-Ser-Δ101</td>
<td>0.7114</td>
<td>0.98891</td>
</tr>
<tr>
<td></td>
<td>0.7432</td>
<td>0.9960</td>
</tr>
</tbody>
</table>

\(^a\) The slopes were obtained from linear fits of the plots of \([\text{Mn(II)}_{\text{free}}]\) versus \([\text{Mn(II)}_{\text{total}}]\). The plots are provided in Figure 4.3.5. Each entry represents an independent titration. \(^b\) Data taken from Hayden, et. al. 2013.\(^4\)
4.3.5. Low-Temperature EPR Spectroscopy Supports the Contribution of the HXH Motif

Simulation of the low-temperature EPR signal for Mn(II) coordinated by CP-Ser (α₂β₂) afforded zero-field splitting parameters consistent with a nearly idealized octahedral coordination sphere \( (D = 270 \text{ MHz}, E/D = 0.30) \). To further probe the Mn(II) coordination sphere, we acquired EPR spectra for samples containing 100 μM of CP-Ser or mutant protein, 30 μM Mn(II), and 1.0 mM Ca(II) (Figure 4.3.6 and Appendix 2).

The Mn(II)-EPR spectra for E96A, D98A, E99A, and E111A exhibit six-line patterns at \( g = 2 \) that closely resemble those for CP-Ser (Figure 4.3.6 panel A). In contrast, there is a dramatic reduction in the signal intensity for CP-Ser-Δ101. The loss of signal intensity and the substructure is comparable to the Mn(II) signals we have reported for the (S100A8)H17A, (S100A8)H27A, (S100A8)H27D (S100A9)H91A, and (S100A9)H95A single mutants reported in Chapter 3.4 Moreover, the spectra of H103A, H105A, CP-Ser-AAA, and CP-Ser-AHA closely resemble the CP-Ser-Δ101 signal. In contrast, the EPR signal of H104A is indistinguishable from CP-Ser. Taken together, these results confirm that His103 and His105 complete the octahedral coordination sphere of Mn(II) at CP site 2 (Figure 4.3.7).

The low-temperature Mn(II) EPR signatures of CP-Ser-AHA(K106H) and CP-Ser-AAA(L109H)(E111H) each exhibit a sharp feature at \( g = 1.88 \) attributed to semi-forbidden transitions; however, the signals are of significantly lower intensity than for CP-Ser (α₂β₂). These data are consistent with the presence of a relatively small amount of six-coordinate Mn(II) in each sample. It is possible that these samples contain mixtures of five- and six-coordinate Mn(II) complexes, which would account for the attenuated six-coordinate EPR signals and ZP1 competition assays, the latter of which support higher-affinity Mn(II) coordination than Δ101 and AAA.
Figure 4.3.6. Low-temperature EPR spectra of CP C-terminal tail mutants (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Low-temperature EPR signals of CP-Ser, E96A, D98A, E99A, E111A, and Δ101. The Δ101 spectrum is scaled by 10x. (B) Low-temperature EPR spectra of CP-Ser, H104A, H103A, H105A, AHA, and AAA. The H103A, H105A, AHA, and AAA spectra are all scaled by 10x. All samples contain 100 μM protein, 1 mM Ca(II), and 30 μM Mn(II). Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.

Figure 4.3.7. The Mn(II) coordination sphere at site 2 of human CP, as supported by crystallographic and solution studies. Mn(II) is coordinated in an octahedral manner by (A8)His17, (A8)His27, (A9)His91, (A9)His95, (A9)His103, and (A9)His105.
4.3.6. The S100A9 HxH Motif is Conserved

Amino acid sequence alignment of human S100A9 with S100A9s from other species reveals a conserved HxH motif in the C-terminal regions of these proteins (Figure 4.3.8 and Appendix 1). To probe whether CP variants containing S100A9 chimeras retain high-affinity Mn(II) coordination, we prepared and characterized CP-Ser-mT (Table 4.3.1). This mutant is a chimera of human and mouse S100A9 where the C-terminal 19 residues of human S100A9 are substituted by the C-terminal 17 residues of the mouse congener. CP-Ser-mT contains a single Cys at position 110, and it was isolated as a Cys110-BME adduct. This adduct was reduced with TCEP, and the resulting protein was buffer exchanged into argon- or nitrogen-purged buffer to remove the BME adduct and prevent undesirable cysteine-based oligomerization.

**Table 4.3.1**

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>human A9</td>
<td>SHEKMEHED E-GPGHHHKP GLGEHTP...</td>
</tr>
<tr>
<td>murine A9</td>
<td>CHEKLEHNNP R-GHHSIGK GCGK...</td>
</tr>
<tr>
<td>rat A9</td>
<td>CHEKLEHNNP R-GHHSIGK GCGK...</td>
</tr>
<tr>
<td>bovine A9</td>
<td>SHEEMHRNTAP P-GQGRHHP GYGKGGSGSC 118 (of 156)</td>
</tr>
<tr>
<td>rabbit A9</td>
<td>SHEEMHNAP HDHEGSHHP GLGGGPGHG 119 (of 132)</td>
</tr>
</tbody>
</table>

**Figure 4.3.8.** Sequence alignment of the C-terminal regions of various S100A9 homologs. The C-terminal HxH motif (underlined) is conserved among S100A9 proteins from multiple species. Full sequence alignments are provided in Appendix 1.

The Mn(II)-binding properties of both CP-Ser-mT and CP-Ser-mT-BME were investigated. The chimeras coordinate Mn(II) with high affinity (see Appendix 2, Figure A2.57) and we see no evidence for participation of Cys110. Moreover, the low-temperature EPR signal of 100 μM CP-Ser-mT incubated with 30 μM Mn(II) exhibit features that are identical to those of CP-Ser (αβ) both in the absence and presence of Ca(II) (Figure 4.3.9). It is noteworthy that the Mn(II) signal for CP-Ser-mT (αβ) exhibits considerably greater intensity and definition compared to that for CP-Ser (αβ), but is less intense than for CP-Ser-mT (αβ). One possible explanation for these subtle differences is that the CP-Ser-mT
dimer coordinates a larger proportion of Mn(II) in a six-coordinate manner compared to CP-Ser. Alternatively, the CP-Ser-mT sample may contain less free Mn(II) in solution, and thus the signal resulting from six-coordinate Mn(II)-bound CP-Ser-mT is undistorted. In total, biochemical and spectroscopic studies of CP-Ser-mT support the notion that His residues within the mouse tail are necessary for high-affinity Mn(II) coordination. Because the mouse tail harbors two adjacent HXH motifs, the identities of the His residues presumed to contribute to Mn(II) binding at site 2 are currently unclear. Moreover, the fact that the CP-Ser-mT heterotetramer retains the ability to coordinate Mn(II) with high affinity, and exhibits a CP-Ser-like EPR spectroscopic signature, indicates that the precise amino acid sequence of the tail region is dispensable at least to some degree. Nevertheless, the amino acid sequence of the tail region may have as-yet unappreciated contributions to metal-ion coordination at site 2, and the heterooligomeric properties of CP homologues, which are largely unexplored, may also influence metal-ion coordination. Further biophysical and structural investigations are required to evaluate the contributions of tail composition and oligomerization state to the coordination sphere and metal-ion affinity at site 2 for CP proteins from different species.
Figure 4.3.9. Low-temperature EPR spectra of the CP-Ser-mT-BME, and CP-Ser-mT. All samples contained 100 µM protein and 30 µM Mn(II) in absence (black line) or presence (red line) of 1 mM Ca(II). Both panels have the same y-axis scale. Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.
4.4 Summary and Perspectives

The results presented in this chapter reveal that CP utilizes an unprecedented biological His$_6$ coordination sphere to coordinate bioavailable Mn(II) in solution. These solution studies are in excellent agreement with structures of Ca(II)- and Mn(II)-bound CP-Ser.$^{5,19}$ The hexahistidine motif is unique among known biological Mn(II) coordination motifs, and Mn(II) coordination at interfacial protein sites is unusual.$^{20}$ Most biological Mn(II) coordination spheres provide a mixture of nitrogen- and oxygen-based ligands. (Figure 4.4.1). Moreover, the primary coordination sphere of the His$_3$Asp site of CP (site 1) is identical to that of human Mn superoxide dismutase;$^{21}$ however, the affinity of site 1 for Mn(II) appears to be in the micromolar range (apparent $K_d$ site 1 = 21 ± 5 μM).$^4$

The acidic residues that are housed in the S100A9 C-terminal tail do not directly coordinate Mn(II), and in general are not conserved across different species (Appendix 1). Nevertheless, these residues may contribute to recruitment of Mn(II) and other metals from solution, or they may contribute to charge neutralization at the Mn(II)-His$_6$ site (further discussion is in Appendix 4). Also of interest from this standpoint is the C-terminal region of human S100A8, which contains a –EESHKE sequence at the C-terminus. These residues do not appear to coordinate Mn(II) or Zn(II) based on crystallographic or solution studies;$^{5,15}$ however, they may provide a low-affinity "trap" for divalent metals in solution and assist in metal ion coordination. Although site 2 is responsible for high-affinity Mn(II) coordination, this site exhibits a thermodynamic preference for Zn(II) coordination,$^{4,15}$ and detailed assays will be required to uncouple the effects of Mn(II) vs. Zn(II) sequestration in vitro and in vivo.
Figure 4.4.1. Examples of mononuclear biological Mn(II)-coordination spheres. (A) The His$_6$ site of human calprotectin$^{5,17}$ (B) The photochemical reaction center of *Rhodobacter sphaeroides*.6 (C) A cupin of unknown function from *Thermotoga maritima*.7 (D) Site 1 of the *Bacillus subtilis* oxalate decarboxylase.8 (E) Site 2 of the *B. subtilis* oxalate decarboxylase.8 (F) The hammerhead ribozyme.9 (G) The His$_3$Asp site of human calprotectin.9,5 (H) The primary coordination sphere of Mn-SOD.21 (I) The TroA solute binding protein from *Streptococcus suis* and *Treponema pallidum*.11,22 The numbering for the *S. suis* TroA is shown. Both TroAs have been crystalized in the Zn(II)-bound forms, but biochemical data indicates that these proteins transport Mn(II). (J) MncA from *Synechocystis* PCC 6803.12 (K) The Mn(II)-solute binding protein of *Staphylococcus aureus*.23 (L) The pneumococcal surface antigen (PsaA) from *Streptococcus pneumoniae* depicted with a four-coordinate geometry.14
Alignment of S100A9s from multiple mammals indicates that the metal-coordinating residues His20, Asp30, His91, His95, His103, and His105 (human numbering) are conserved. The cow, rabbit, horse, sheep, cat, and dog sequences harbor additional C-terminal regions that are rich in glycine and histidine (Appendix 1). For example the bovine S100A9 is a 156 amino acid protein (ca. 17 kDa) that has been isolated from neutrophil cytosol. The C-terminal region of bovine S100A9 houses the conserved HxH motif (Figure 4.3.8) and harbors an additional 12 His residues in a His- and Gly-rich region. It is unclear how these His-rich regions influence metal coordination, or if they provide additional coordination spheres.

Acknowledgements

Protein purification and experiments were performed in collaboration with Ms. Aleth Gaillard and Mr. Toshiki Nakashige. Aleth Gaillard prepared and characterized CP-Ser(D98A), CP-Ser(E96A), and CP-Ser(E99A), and Toshiki Nakashige prepared and characterized CP-Ser-AHA(K106H), CP-Ser-AAA(K106H), and CP-Ser-AAA(L109H)(E111H). The thermal denaturation experiments presented in this chapter were performed by Aleth Gaillard. The Searle Scholars Program (Kinship Foundation), the MIT Center for Environmental Health Sciences (NIH P30-ES002109), the MIT Department of Chemistry, the Stephen J. Lippard Fellowship Fund (summer graduate fellowship to MBB), and the ENS de Cachan (internship support to AG) are gratefully acknowledged for financial support. We thank Dr. Ralph Weber, Dr. Jeffrey Simpson, and Alexandria Liang for assistance with the EPR spectrometer located in the MIT Department of Chemistry Instrumentation Facility and Debby Pheasant for assistance with the CD spectrometer. The CD spectrometer is housed in the MIT Biophysical Instrumentation Facility for the Study of Complex Macromolecular Systems, which is supported by grants NSF 0070319 and NIH GM68762.
References


Soc. 2006, 128, 16764.


(23) Gribenko, A.; Mosyak, L.; Ghosh, S.; Parris, K.; Svenson, K.; Moran, J.; Chu, L.; Li,

Appendix 1: Sequence Alignments of Transition Metal Binding S100 Proteins
Figure A1.1. Sequence alignment of human S100A7 and S100A15 with related sequences. Zn(II)-coordinating residues are colored red, and disulfide-forming cysteines are colored green. Human S100A15 is the only entry that does not encode an aspartate residue at position 25. All other entries are annotated as S100A7 proteins. The Uniprot accession numbers, as well as the species, for each amino acid sequence are given.
Figure A1.2. Sequence alignment of human S100A8 with other mammalian orthologs. Zinc(II)- and manganese(II)-coordinating residues are colored orange. The human sequence houses an additional 4 amino acids (EESH) at the C-terminus. The Uniprot or PubMed (sheep, cat, and dog) accession numbers, as well as the species, for each amino acid sequence are given.
Figure A1.3. Sequence alignment of human S100A9 with mammalian orthologs. Zinc(II)-and manganese(II)-coordinating residues are colored orange.\textsuperscript{2-5} The pig, cow, rabbit, horse, cat, and dog sequences harbor an additional 16-42 amino acid region that is rich in glycine and histidine. The Uniprot or Pubmed (cat and dog) accession numbers, as well as the species, for each amino acid sequence are given.
Figure A1.4. Sequence alignment of human S100A12 with mammalian orthologs. Zinc(II) and copper(II)-coordinating residues are colored red. These sequences contain ca. 50% overall sequence identity. The Uniprot accession numbers, as well as the species, for each amino acid sequence are given.
| Q6513 | Mouse | MPDPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DSVPRFMDTL GRRQPYYITE 60 |
| D3ZM0 | Rat    | MPDPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DSVPRFMDTL GRRQPYYITE 60 |
| G31880 | Chinese hamster | MPDPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DSVPRFMDTL GRRQPYYITE 60 |
| E18PR8 | Cow    | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| H2N5P5 | Orangutan | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| F6QD1 | Rhesus macaque | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| F6WG34 | Horse  | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| A7K6Y8 | Gorilla | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| I31024 | Squirrel | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| G1Q2A6 | Brown bat | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| GITIX1 | Rabbit | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| H0Y2B7 | Galago  | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| H0W352 | Guinea pig | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| M31X3 | Cat | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| E2RP44 | Dog | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| F1SU56 | Pig | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| W5NQM2 | Sheep | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| F6SKM4 | Opossum | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |
| L35JV8 | Fox | MDTPVESSL FQIIHCFHY AAREGDKETL SLEELKALLL DNVPFRMGETL GRRQPYYITE 60 |

**Figure A1.5.** Sequence alignment of mouse S100A15 with mammalian homologs. Residues that are predicted to coordinate transition metal-ions based on sequence alignment with human S100A9 are colored red. Cysteine residues are colored green. Murine S100A15 has not been characterized biochemically, and its Ca(II) / Zn(II) / Mn(II) binding properties, as well as any potential redox properties, have not been studied. The Uniprot accession numbers, as well as the species, for each amino acid sequence are given.
Figure A1.6. Sequence alignment of human S100B with homologs from various mammals as well as Atlantic salmon. Zinc(II)-coordinating residues are colored red. The Uniprot accession numbers, as well as the species, for each amino acid sequence are given.
References


Appendix 2: Additional Characterization of CP and Mutant Proteins

The figures in this appendix are reproduced or adapted from:


## Calprotectin Protein Nomenclature

### Table A2.1. Calprotectin protein nomenclature.

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<td>(C3S) with murine C-terminal tail</td>
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Circular Dichroism Spectroscopy

Figure A2.1. CD spectra of 10 μM CP and 10 μM CP-Ser in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.2. CD spectra of 10 µM CP-Ser(H20A) and 10 µM CP-Ser(D30A) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C). The data was only collected to 200 nm because of high dynode at shorter wavelengths.
**Figure A2.3.** CD spectra of 10 µM CP-Ser(H83A) and 10 µM CP-Ser(H87A) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.4. CD spectra of 10 μM CP-Ser(H17A) and 10 μM CP-Ser(H27A) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.5. CD spectra of 10 μM CP-Ser(H91A) and 10 μM CP-Ser(H95A) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.6. CD spectra of 10 µM CP-Ser(D30A)(H83A) and 10 µM CP-Ser(H27A)(H91A) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.7. CD spectra of 10 µM CP-Ser-ΔHis₃Asp and 10 µM CP-Ser-ΔHis₄ in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.8. CD spectra of 10 μM CP-Ser-ΔΔ and 10 μM CP-Ser(H27D) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.9. CD spectra of 10 μM CP-Ser(E96A) and 10 μM CP-Ser(D98A) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.10. CD spectra of 10 µM CP-Ser(E99A) and 10 µM CP-Ser(H103A) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.11. CD spectra of 10 μM CP-Ser(H104A) and 10 μM CP-Ser(H105A) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.12. CD spectra of 10 μM CP-Ser(K106A) and 10 μM CP-Ser(E111A) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.13. CD spectra of 10 μM CP-Ser-AHA and 10 μM CP-Ser-AAA in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.14. CD spectra of 10 μM CP-Ser-AHA and 10 μM CP-Ser-AAA in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.15. CD spectra of 10 μM CP-Ser-AHA(K106H) and 10 μM CP-Ser-AAA(L109H)(E111H) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.16. CD spectra of 10 μM CP-Ser-Δ101 and 10 μM CP-Ser-mT-BME in the absence (black circles) and presence (red squares) of 2 mM Ca(II) at pH 8.5 (1 mM Tris, 0.5 mM EDTA; T = 25 °C).
Figure A2.17. Analytical size exclusion chromatography of 100 μM CP in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. The red stars indicate Ca(II)-dependent peaks attributed to higher order oligomers. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.18. Analytical size exclusion chromatography of 100 μM CP-Ser in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. The red star indicates Ca(II)-dependent peaks attributed to higher order oligomers. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.19. Analytical size exclusion chromatography of 100 μM CP-Ser(H17A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. The red star indicates Ca(II)-dependent peaks attributed to higher order oligomers. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.20. Analytical size exclusion chromatography of 100 µM CP-Ser(H27A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. The red star indicates Ca(II)-dependent peaks attributed to higher order oligomers. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.21. Analytical size exclusion chromatography of 100 μM CP-Ser(H91A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.22. Analytical size exclusion chromatography of 100 μM CP-Ser(H95A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
**Figure A2.23.** Analytical size exclusion chromatography of 100 μM CP-Ser(H27A)(H91A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. The red star indicates Ca(II)-dependent peaks attributed to higher order oligomers. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.24. Analytical size exclusion chromatography of 100 µM CP-Ser ΔHis₄ in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. The red stars indicate Ca(II)-dependent peaks attributed to higher order oligomers. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.25. Analytical size exclusion chromatography of 100 μM CP-Ser(H83A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
**Figure A2.26.** Analytical size exclusion chromatography of 100 μM CP-Ser(H87A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.27. Analytical size exclusion chromatography of 100 μM CP-Ser(H20A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.28. Analytical size exclusion chromatography of 100 μM CP-Ser(D30A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.29. Analytical size exclusion chromatography of 100 µM CP-Ser(D30A)(H83A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.30. Analytical size exclusion chromatography of 100 μM CP-Ser ΔHis₃Asp in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.31. Analytical size exclusion chromatography of 100 μM CP-Ser ΔΔ in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 2.3.2.
Figure A2.32. Analytical size exclusion chromatography of 200 μM CP-Ser(H27D) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature.
Figure A2.33. Analytical size exclusion chromatography of 100 μM CP-Ser(E96A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.34. Analytical size exclusion chromatography of 100 μM CP-Ser(D98A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.35. Analytical size exclusion chromatography of 100 μM CP-Ser(E99A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.36. Analytical size exclusion chromatography of 100 μM CP-Ser(H103A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.37. Analytical size exclusion chromatography of 100 μM CP-Ser(H104A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.38. Analytical size exclusion chromatography of 100 μM CP-Ser(H105A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.39. Analytical size exclusion chromatography of 100 μM CP-Ser(K106A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.40. Analytical size exclusion chromatography of 100 μM CP-Ser(E111A) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.41. Analytical size exclusion chromatography of 100 μM CP-Ser-AHA in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.42. Analytical size exclusion chromatography of 100 μM CP-Ser-AAA in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.43. Analytical size exclusion chromatography of 100 μM CP-Ser-AAE in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.44. Analytical size exclusion chromatography of 100 μM CP-Ser-AAA(K106H) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.45. Analytical size exclusion chromatography of 100 µM CP-Ser-AHA(K106H) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.46. Analytical size exclusion chromatography of 100 μM CP-Ser-AAA(L109H)(E111H) in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.47. Analytical size exclusion chromatography of 100 μM CP-Ser-Δ101 in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Figure A2.48. Analytical size exclusion chromatography of 100 µM CP-Ser-mT in the absence (black) and presence (red) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (A) Full chromatograms. (B) Expansion. Absorption was monitored at 280 nm at room temperature. Elution volumes are listed in Table 4.3.3.
Manganese(II) Analytical Size Exclusion Chromatography

Figure A2.49. Analytical SEC of CP-Ser and mutant proteins in the presence of Mn(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). The red traces are CP-Ser and mutants in the presence of 10 equivalents of Mn(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). The black traces are standards of CP-Ser in the absence and presence of 2 mM Ca(II). The protein concentrations were 100 μM (100 μL, black traces) and 200 μM (200 μL, red traces). In each panel, the vertical lines indicate the elution volumes for the CP-Ser αβ and α2β2 forms.
Figure A2.50. Analytical SEC of CP-Ser-AHA (A), CP-Ser-AAA (B), CP-Ser-AAE (C) and CP-Ser-AAA(K106H) (D) in the presence of Mn(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). The black traces are standards of the indicated mutant in the absence and presence of 2 mM Ca(II). The red traces are the CP-Ser mutants in the presence of 10 equivalents of Mn(II) after a 0, 2 and 18 h incubation time. The protein concentrations were 100 µM (100 µL, black traces) and 200 µM (200 µL, red traces). The vertical lines indicate the elution volumes for the αβ and αβ2 forms of the indicated mutant.
Figure A2.51. Analytical SEC of CP-Ser-AHA(K106H) (A), CP-Ser-AAA(L109H)(E111H) (B) and CP-Ser-Δ101 (C) in the presence of Mn(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). The black traces are standards of the indicated mutant in the absence and presence of 2 mM Ca(II). The red traces are the CP-Ser mutants in the presence of 10 equivalents of Mn(II) after a 0, 2 and 18h incubation time. The protein concentrations were 100 μM (100 μL, black traces) and 200 μM (200 μL, red traces). The vertical lines indicate the elution volumes for the $\alpha\beta$ and $\alpha_2\beta_2$ forms of the indicated mutant.
Figure A2.2. Competition between ZP1 and CP-Ser(E96A) (A), CP-Ser(D98A) (B), and CP-Ser(E99A) (C) for Mn(II). All titrations were conducted with 1 μM ZP1, 4 μM CP, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The integrated emission of apo ZP1 was normalized to a value of one.
Figure A2.53 Competition between ZP1 and CP-Ser(H103A) (A), CP-Ser(H104A) (B), and CP-Ser(H105A) (C) for Mn(II). All titrations were conducted with 1 μM ZP1, 4 μM CP, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The integrated emission of apo ZP1 was normalized to a value of one.
Figure A2.54. Competition between ZP1 and CP-Ser(K106A) (A), CP-Ser(E111A) (B), and CP-Ser-AHA (C) for Mn(II). All titrations were conducted with 1 µM ZP1, 4 µM CP, and 200 µM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The integrated emission of apo ZP1 was normalized to a value of one.
Figure A2.55. Competition between ZP1 and CP-Ser-AAA (A), CP-Ser-AAE (B), and CP-Ser-AAA-(K106H) (C) for Mn(II). All titrations were conducted with 1 μM ZP1, 4 μM CP, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The emission of apo ZP1 was normalized to a value of one.
Figure A2.56. Competition between ZP1 and CP-Ser-AHA(K106H) (A), CP-Ser-AAA(L109H)(E111H) (B), and CP-Ser-A101 (C) for Mn(II). All titrations were conducted with 1 μM ZP1, 4 μM CP, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The emission of apo ZP1 was normalized to a value of one.
Figure A2.57. Competition between ZP1 and CP-Ser-mT-BME (A) and CP-Ser-mT (B) for Mn(II). All titrations were conducted with 1 μM ZP1, 4 μM CP, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The emission of apo ZP1 was normalized to a value of one.
Figure A2.58. Low-temperature EPR spectra of the CP mutant proteins E96A, D98A, E99A, H103A, H104A, and H105A. All samples contained 100 µM protein and 30 µM Mn(II) in absence (black line) or presence (red line) of 1 mM Ca(II). All panels contain the same y-axis scale. Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.
Figure A2.59. Low-temperature EPR spectra of the CP mutant proteins K106A, E111A, AHA, AAA, AAE, and AAA(K106H). All samples contained 100 μM protein and 30 μM Mn(II) in absence (black line) or presence (red line) of 1 mM Ca(II). All panels contain the same y-axis scale as used in Figure A2.58. Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.
Figure A2.60. Low-temperature EPR spectra of the CP mutant proteins AHA(K106H), AAA(L109H)(E111H) and Δ101. All samples contained 100 μM protein and 30 μM Mn(II) in absence (black line) or presence (red line) of 1 mM Ca(II). All panels contain the same y-axis scale as used in Figures A2.58 and A2.59. Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.
Appendix 3: Structures of Colorimetric and Fluorescent Sensors Employed in this Work
Figure A3.1. Structure of apo Zincon

Figure A3.2 Structure of apo MagFura-2
Figure A3.3. Structure of apo Zinpyr-1

Figure A3.4. Structure of apo Zinpyr-4
Appendix 4: Manganese Binding Properties of Human Calprotectin under Conditions of High and Low Calcium

This appendix is reproduced from *J. Am. Chem. Soc.* **2015**, *137*, 3004-3016. Samples for crystallization and electron paramagnetic resonance spectroscopy were prepared by Megan Brophy. Diffraction data were collected by MB and Sarah Bowman, and all diffraction data was analyzed by SB. EPR data were collected, processed, and analyzed by Derek Gagnon. The paper was written by DG, MB, SB, and Liz Nolan.
Manganese Binding Properties of Human Calprotectin Under Conditions of High and Low Calcium: X-ray Crystallographic and Advanced EPR Spectroscopic Analysis

Derek M. Gagnon, Megan Brunjes Brophy, Sarah E. J. Bowman, Troy A. Stich, Catherine L. Drennan, R. David Britt, and Elizabeth M. Nolan

1Department of Chemistry, University of California Davis, Davis, CA 95616, United States
2Department of Chemistry, 3Howard Hughes Medical Institute, and 4Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, United States

*Correspondence:
Inolan@mit.edu
Phone: 617-452-2495
Fax: 617-324-0505
rdbritt@ucdavis.edu
Phone: 530-752-6377
Fax: 530-752-8995
Abstract

The antimicrobial protein calprotectin (CP), a hetero-oligomer of the S100 family members S100A8 and S100A9, is the only identified mammalian Mn(II)-sequestering protein. Human CP uses Ca(II) ions to tune its Mn(II) affinity at a biologically unprecedented hexahistidine site that forms at the S100A8/S100A9 interface, and the molecular basis for this phenomenon requires elucidation. Herein, we investigate the remarkable Mn(II) coordination chemistry of human CP using X-ray crystallography as well as continuous wave (CW) and pulse electron paramagnetic resonance (EPR) spectroscopies. An X-ray crystallographic structure of Mn(II)-CP containing one Mn(II), two Ca(II), and two Na(I) ions per CP heterodimer is reported. The CW EPR spectrum of Ca(II)- and Mn(II)-bound CP prepared with a 10:0.9:1 Ca(II):Mn(II):CP ratio is characterized by an unusually low zero-field splitting of 485 MHz (E/D = 0.30) for the S = 5/2 Mn(II) ion, consistent with the high symmetry of the His$_6$ binding site observed crystallographically. Results from electron spin-echo envelope modulation and electron nuclear double resonance experiments reveal that the six Mn(II)-coordinating histidine residues of Ca(II)- and Mn(II)-bound CP are spectroscopically equivalent. The observed $^{15}$N ($I = 1/2$) hyperfine couplings ($A$) arise from two distinct classes of nitrogen atoms: the coordinating $\varepsilon$-nitrogen of the imidazole ring of each histidine ligand ($A = [3.45, 3.71, 5.91]$ MHz) and the distal $\delta$-nitrogen ($A = [0.11, 0.18, 0.42]$ MHz). In the absence of Ca(II), the binding affinity of CP for Mn(II) drops by ca. two orders of magnitude and coincides with Mn(II) binding at the His$_6$ site as well as other sites. This study demonstrates the role of Ca(II) in enabling high-affinity and specific binding of Mn(II) to the His$_6$ site of human calprotectin.
A4.1 Introduction

Competition between the mammalian host and bacterial pathogens for the essential metal nutrient Mn(II) is an important facet of the innate immune response and bacterial pathogenesis.\textsuperscript{1–4} Animal models of infectious disease indicate that various human pathogens, including \textit{Staphylococcus aureus} and \textit{Streptococcus pneumoniae}, must acquire Mn(II) in order to establish an infective lifestyle.\textsuperscript{1,5,6} The mammalian innate immune system employs the Ca(II)-, Mn(II)-, and Zn(II)-binding protein calprotectin (CP) in a metal-withholding strategy that limits Mn(II) and Zn(II) availability at sites of infection.\textsuperscript{7,8} CP is expressed and released by neutrophils and epithelial cells, and exhibits broad-spectrum antimicrobial activity attributed to its metal-binding properties.\textsuperscript{7–13} Moreover, CP is currently the only host-defense protein identified that sequesters labile Mn(II). Human CP accomplishes this feat by using a biologically unprecedented hexahistidine coordination motif.\textsuperscript{10,11,13}

CP employs Ca(II) ions to modulate its affinities for Mn(II) and Zn(II).\textsuperscript{9,10} In our working model, CP turns on its metal-sequestering ability in the Ca(II)-rich (i.e. low-millimolar concentrations) extracellular space such that it can outcompete invading pathogens for these transition metal ions. Nevertheless, the molecular basis for how Ca(II) ions enable CP to transition from a relatively low- to a relatively high-affinity form, as well as how the unusual hexahistidine site allows CP to entrap labile Mn(II), requires elucidation. In the present work, we employ X-ray crystallography and electron paramagnetic resonance (EPR) spectroscopy to interrogate the mechanism by which Ca(II) ions modulate complexation of Mn(II) by CP.

CP is a member of the Ca(II)-binding S100 protein family. S100 proteins employ helix-loop-helix EF-hand domains for Ca(II) coordination, and several members of this family (e.g. S100A7, S100A12, S100B, CP) exhibit additional binding sites for transition metal
ions. CP forms heterodimers and tetr ảnhs of S100A8 (93 amino acids, 10.8 kDa, α) and S100A9 (114 amino acids, 13.2 kDa, β). Whether CP exists as the αβ or αββ2 oligomer depends on Ca(II). In the absence of Ca(II), the αβ heterodimer predominates whereas Ca(II) binding results in formation of the αββ2 tetramer.

<table>
<thead>
<tr>
<th>EF-hand</th>
<th>Ca(II)-coordinating groups</th>
<th>Coordination number</th>
<th>Net charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8, N-terminal Non-canonical A8</td>
<td>Ser20 (C=O), Lys23 (C=O), Asn25 (C=O), Ala28 (C=O), H2O</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>C-terminal Canonical A8</td>
<td>Asp59 (Oδ), Asn61 (Oδ), Asp63 (Oδ1), Ala65 (C=O), Glu70 (Oε1 and Oε2), H2O</td>
<td>7</td>
<td>-4</td>
</tr>
<tr>
<td>A9, N-terminal Non-canonical A9</td>
<td>Ser23 (C=O), Leu26 (C=O), His28 (C=O), Thr31 (C=O), Glu36 (Oε1), H2O</td>
<td>6</td>
<td>-1</td>
</tr>
<tr>
<td>C-terminal Canonical A9</td>
<td>Asp67 (Oδ), Asn69 (Oδ), Asp71 (Oδ1), Gln73 (C=O), Glu78 (Oε1 and Oε2), H2O</td>
<td>7</td>
<td>-4</td>
</tr>
</tbody>
</table>

* Taken from the crystal structure of the Ca(II)-bound CP-Ser heterotetramer (PDB 1XK4, reference 18). C=O indicates coordination by the carbonyl oxygen of the peptide backbone. In the 1XK4 structure, the Ca(II) ions are modeled at 100% occupancy in the canonical C-terminal EF-hand sites, and the Ca(II) ions in the non-canonical N-terminal EF-hand sites are modeled at 70% occupancy.

The Ca(II)-binding sites of CP have been structurally characterized. Each S100 subunit exhibits two EF-hand domains (Table 1), and each subunit has been proposed to coordinate two Ca(II) ions such that the CP heterodimer contains four distinct Ca(II)-binding sites. The C-terminal EF-hand of each subunit resembles the Ca(II)-binding domains of calmodulin and is therefore described as canonical. The N-terminal EF-hands exhibit lower coordination numbers and are often termed non-canonical. The Ca(II)-coordinating groups of S100A8 and S100A9 identified in prior crystallographic structural work, as well as the coordination numbers and net charges of these EF-hands, are...
summarized in Table 1. The Ca(II)-binding affinities of the CP EF-hands have not been
determined; however, Ca(II) affinities of other S100 proteins have been reported.\cite{2,22,24,25} In
general, the reported dissociation constant ($K_d$) values for Ca(II) of canonical EF-hands are
1-50 μM whereas the $K_d$ values for non-canonical EF-hands are 100-500 μM. The relatively
weak Ca(II) affinities of non-canonical EF-hands are consistent with both the lower
coordination numbers and the reduced negative charge exhibited by these motifs. Moreover,
it has been proposed that the Ca(II) affinities of the N-terminal non-canonical EF-hands may
be too weak to appreciably bind Ca(II) in a physiological context.\cite{22}

Each CP heterodimer contains two transition-metal-ion binding sites at the dimer
interface that are distinct from the Ca(II)-binding sites.\cite{9,18} Site 1 is a His$_3$Asp motif comprised
of His83 and His87 from S100A8, and His20 and Asp30 from S100A9. Site 2 is an unusually
histidine-rich biological metal-binding site and has been described as a His$_4$ or His$_6$
motif.\cite{10,11,13} The His$_4$ motif, which is observed crystallographically at the S100A8/S100A9
interface in the absence of a bound transition metal ion,\cite{18} contains His17 and His27 of
S100A8 and His91 and His95 of S100A9. The S100A9 subunit has an extended C-terminal
tail that is disordered in the absence of a coordinated Mn(II) ion. Structural\cite{13} and
biochemical\cite{11} studies of Mn(II)-bound CP revealed that site 2 binds Mn(II) in an octahedral
His$_6$ motif in which His103 and His105 of the S100A9 tail provide the fifth and sixth ligands.
Both sites 1 and 2 can coordinate Mn(II), and we previously identified site 2 as the high-
affinity Mn(II)-binding site.\cite{10} Moreover, we proposed that encapsulation of Mn(II) by the tail
region allows CP to bind and restrict dissociation of this labile metal ion.\cite{11}

In this work, we probe the molecular basis for Mn(II) sequestration by CP using X-ray
crystallography and EPR spectroscopy. We present a new crystallographic snapshot of
Mn(II)-CP that reveals a 1:1 Mn(II):CP ($\alpha\beta$) stoichiometry with Mn(II) exclusively bound to
the His$_6$ site and Ca(II) bound to each of the two canonical C-terminal EF-hands of the $\alpha\beta$
heterodimer. We conclude that the non-canonical EF-hands located in the N-terminal regions of S100A8 and S100A9 coordinate Na(I) under the crystallographic conditions used in this work. Moreover, we establish that the Mn(II)-His$_6$ site of Ca(II)-bound CP is highly symmetric in solution, and that the C-terminal tail region of S100A9 is essential for preventing water access to the Mn(II) center. We demonstrate, for the first time, that Mn(II) binding in the absence of Ca(II) results in Mn(II) coordination to the His$_6$ site as well as additional sites. These findings indicate that Ca(II) binding leads to specific and high-affinity Mn(II) coordination to the His$_6$ site. In total, our work illuminates the molecular basis for Mn(II) capture by CP in the presence of Ca(II), and supports a model in which Ca(II) binding promotes Mn(II) sequestration in an octahedral Mn(II)-His$_6$ coordination environment that precludes water molecules from the primary coordination sphere.
A4.2 Experimental Section

General Materials and Methods. All solvents, reagents, and chemicals were obtained from commercial suppliers and used as received. All buffers and metal stock solutions were prepared using Milli-Q water (18.2 MΩ, 0.22-μm filter). All buffers employed for spectroscopy samples were prepared in acid-washed volumetric glassware using Ultrol grade HEPES (free acid, Calbiochem) and TraceSELECT NaCl (Sigma). PEG 200 was purchased from Hampton Research. Calcium chloride was purchased from Sigma Aldrich, and 99.999% manganese chloride was purchased from Alfa Aesar. Stock solutions of 1.0 M Ca(II) or Mn(II) were prepared in Milli-Q water using acid-washed volumetric glassware and immediately transferred to polypropylene tubes for long-term storage. Working metal solutions were prepared fresh daily by diluting the appropriate stock solution with buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). \(^{15}\)N-Ammonium chloride (\(^{15}\)NH\(_4\)Cl, 99%) and deuterium oxide (\(^2\)H\(_2\)O, 99.9%) were obtained from Cambridge Isotope Laboratories, INC (Andover, MA). Table S1 (Supporting Information) lists the proteins used in this work and corresponding nomenclature. Unlabeled CP-Ser, \(\Delta\)His\(_2\)Asp, H103A, H104A, H105A, H103A/H105A (AHA), and H103A/H104A/H105A (AAA) were overexpressed and purified as described previously.\(^9\) CP-Ser and mutants thereof were employed in this work because mutation of Cys42 of S100A8 and Cys3 of S100A9 to Ser residues allows metal-binding studies to be performed in the absence of a reducing agent.\(^9\) All protein concentrations are reported in terms of the \(\alpha\beta\) heterodimer. Equivalents of divalent cations are reported in terms of the \(\alpha\beta\) heterodimer.

Preparation of \(^{15}\)N-labeled CP-Ser and Mutants. Globally \(^{15}\)N-labeled S100A8(C42S), S100A8(C42S)(H83A)(H87A), S100A9(C3S), and S100A9(C3S)(H20A)(D30A) were obtained by overexpression in M9 minimal medium containing 6 g/L anhydrous Na\(_2\)H\(_2\)PO\(_4\), 3 g/L anhydrous KH\(_2\)PO\(_4\), 0.5 g/L NaCl, 2 mM MgSO\(_4\),
100 μM CaCl₂, 1 g/L ¹⁵NH₄Cl, and 2 g/L D-glucose. The medium was aseptically filtered, and
a 1-L volume was transferred to an autoclaved 4-L baffled flask. The minimal medium (1 L)
was supplemented with a 400-μL aliquot of a vitamin mix (400 mg choline chloride, 500 mg
folic acid, 1.1 g pantothenic acid, 500 mg nicotinamide, 500 mg myo-inositol, 500 mg
pyridoxal HCl, 500 mg thiamine HCl, and 50 mg riboflavin suspended in 15 mL of Milli-Q
water) and a spatula tip of iron(III) chloride hexahydrate immediately prior to use.

The plasmids pET41a-S100A9(C3S), pET41a-S100A8(C42S), pET41a-S100A9
(C3S)(H20A)(D30A), and pET41a-S100A8(C42S)(H83A)(H87A) were transformed into
chemically-competent E. coli BL21(DE3) cells. Single colonies were selected and utilized to
inoculate 50 mL of Luria Broth (LB) medium containing kanamycin (50 μg/mL). Cultures
were incubated overnight (ca. 16 h) at 37 °C with shaking at 175 rpm in 250-mL baffled
flasks. The cultures were transferred to sterile 50-mL conical tubes and pelleted by
centrifugation (3000 rpm x 30 min, 4 °C). The supernatant was decanted, and each cell
pellet was washed by resuspending the cells with ca. 25 mL of minimal medium (vide supra)
and pelleting by centrifugation (3000 rpm x 30 min, 4 °C). The resulting cell pellet was
resuspended in ca. 25 mL of minimal medium and the entire volume was used to inoculate 1
L of minimal medium prepared as described above. The cultures were incubated at 37 °C
with shaking at 175 rpm and protein overexpression was induced by addition of 500 μM of
IPTG when the OD₆₀₀ reached ca. 0.6. The cultures were incubated for an additional 3.5 h at
37 °C (OD₆₀₀ ~ 1.2) at which time the cells were harvested by centrifugation (3000 rpm x 15
min, 4 °C). The cell pellets were transferred to sterile 50-mL conical tubes, flash frozen in
liquid nitrogen, and stored at -80 °C for up to six weeks. The resulting yields ranged from
approximately 1.0–2.0 g cells / L of culture.

Globally ¹⁵N-labeled CP-Ser and ΔHis₃Asp were purified and stored as described
previously, and the yields ranged from 3-7 mg of ¹⁵N-labeled protein / 2 L of culture. For
mixed-labeled proteins where only S100A8 or S100A9 was $^{15}$N-labeled, the unlabeled subunit was overexpressed in M9 minimal medium containing NH$_4$Cl with natural abundance. For all protein purifications, the final size-exclusion chromatography and dialysis steps were performed at pH 8.0 (20 mM HEPES, 100 mM NaCl) as described previously,$^9$ and the purified proteins were stored at -80 °C until further use. Protein purity and identity were confirmed by SDS-PAGE (Figure S1) and LC/MS (Table S2).

**Crystallization of Mn(II)-CP-Ser.** Mn(II)-CP-Ser was crystallized at 18 °C employing the sitting drop vapor diffusion technique. Screening of crystallization conditions using an automated Phoenix crystallization robot (Art Robbins Instrument) and Crystal Screen HT (Hampton Research) yielded a crystal that appeared between days 3 and 5. The droplets contained 100 nL of protein solution, 100 μM CP-Ser, 100 μM MnCl$_2$, 1.0 mM CaCl$_2$, at pH 7.5 (75 mM HEPES, 100 mM NaCl) and 100 nL of precipitant solution, 20% (w/v) PEG 4000, 20% (v/v) isopropanol, 0.1 M sodium citrate at pH 5.6, and were equilibrated against 100 μL of precipitant solution in a 96-well Intelliplate (Art Robbins Instruments). The crystal was transferred to a cryogenic solution (20% PEG 4000, 10% glycerol) and flash-frozen in liquid nitrogen.

**Data Collection and Structure Determination.** Diffraction data were collected at the 24-ID-C beamline at the Advanced Photon Source (APS) at Argonne National Laboratory using a Pilatus 6M pixel detector. APS datasets were collected at a wavelength of 0.9792 Å (12662.0 eV) in 0.3° oscillation steps. Additional diffraction data was collected on the same crystal at 100 K using an in-house Cu-K$_\alpha$ rotating anode source (Rigaku) at a wavelength of 1.5418 Å with a Saturn 944 CCD detector, using sequential 1° oscillation steps. The structure was completed with SBGrid compiled software.$^{26}$ Data were integrated and scaled in space group $P2_1$ ($a = 55.2$ Å, $b = 49.2$ Å, $c = 218.1$ Å, $\alpha = \gamma = 90$ and $\beta = 94.1$), using HKL2000 software.$^{27}$ Two and a half tetramers are contained within the asymmetric
The structure was determined at 2.5 Å resolution by molecular replacement using protein atoms from chains A and C of PDB entry 4GGF in PHASER. Iterative rounds of refinement in PHENIX without use of noncrystallographic symmetry restraints or a sigma cutoff, and model building in COOT achieved a final model at 1.76 Å resolution. Initial difference electron density maps revealed density consistent with the presence of five Mn(II) in the five His₆ sites, 10 Ca(II) ions in the 10 canonical C-terminal EF-hand sites, and 10 ions in the 10 non-canonical N-terminal EF-hand sites of the asymmetric unit. To further probe the identity of the metals in the metal-binding sites, in-house diffraction data, processed using HKL2000, was employed to generate anomalous density maps. The anomalous data are consistent with five Mn(II) and 10 Ca(II) ions in the asymmetric unit. There is no anomalous density observed at the non-canonical EF-hand sites. To best fit the electron density in these sites, and yield B-factors similar to the rest of the protein, Na(I) is modeled into both the S100A8 and S100A9 non-canonical N-terminal EF-hand sites at 100% occupancy. The crystallization conditions contained 100 mM NaCl in the protein solution (100-fold excess relative to CP-Ser). Metal ions and water molecules were added to the model at 2.0-Å resolution. The model contains 8921 total atoms; chains C and E contain residues 1-89 (of 93, S100A8), chains A, G, and I contain residues 1-88 (of 93, S100A8), chains B, F, and J contain residues 4-111 (of 114, S100A9), and chains D and H contain residues 4-112 (of 114, S100A9). Composite omit maps were used to verify the structure. The crystallographic data refinement and statistics are provided in Table S3.

**Preparation of EPR Samples.** Aliquots of CP-Ser or mutated forms of CP-Ser were thawed on ice and buffer exchanged into 75 mM HEPES, 100 mM NaCl, pH 7.5. The protein concentration was determined by absorbance at 280 nm (ε = 18,450 M⁻¹ cm⁻¹ for CP-Ser (αβ) and mutants presented herein). For +Ca(II) EPR samples, a solution (150 μL) of 200 μM CP at pH 7.5 (80% (v/v) 75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200) was
prepared in a microcentrifuge tube, and Ca(II) was added from a 100-mM stock solution to afford a final concentration of 2 mM. The resulting mixture was incubated at room temperature for ca. 20 min. For +Ca(II) samples, Mn(II) was then added from a 10-mM stock solution to afford a final concentration of 180 μM (0.9 equiv. / CP αβ), and the sample was incubated at room temperature for 20 min. The sample was then transferred to a quartz (CFQ) EPR tube with an outer diameter of 3.8 mm (Wilmad, 706-PQ-9.50), frozen in liquid nitrogen, and stored in liquid nitrogen.

For +Ca(II) samples prepared in 2H2O, protein aliquots were thawed and buffer exchanged as described above, and CP was diluted to 100 μM (300 μL) at pH 7.5 (75 mM HEPES, 100 mM NaCl). An aliquot of aqueous Ca(II) (100 mM) was added to each sample to afford a final Ca(II) concentration of 1 mM (10 equiv. / αβ), and the resulting sample was incubated at room temperature for ca. 20 min. An aliquot of Mn(II) (10 mM) was then added to achieve a final concentration of 90 μM (0.9 equiv. / CP αβ) and the sample was incubated at room temperature for ca. 20 min. The resulting samples were frozen in liquid nitrogen and lyophilized to dryness. To exchange H2O for 2H2O, each sample was re-suspended in 300 μL of 2H2O and lyophilized to dryness. This process was repeated for a total of three times. Following the third lyophilization, each sample was dissolved in 150 μL of 2H2O containing 20% (v/v) PEG 200 to afford a sample containing 200 μM CP, 2 mM Ca(II), and 180 μM Mn(II). Each sample was transferred to an EPR tube, frozen in liquid nitrogen, and stored in liquid nitrogen. For experiments at Q-band, several samples were thawed and transferred into quartz EPR tubes (2.4 x 2.0 mm), and frozen in liquid nitrogen.

The –Ca(II) samples were prepared as described above except that samples contained 180 μM Mn(II) and either 1.0 mM or 200 μM CP. No Ca(II) was added.

**Electron Paramagnetic Resonance Spectroscopy.** EPR spectra were collected at the CalEPR Center at the University of California, Davis. All X-band (ca. 9.4 GHz)
continuous wave (CW) EPR spectra were collected under non-saturating, slow-passage conditions using a Bruker E500 equipped with a super high QE (SHQE) resonator and Oxford Instruments ESR900 cryostat. The time constant was set to 80 ms with a data point collected ca. every 0.4 mT. A Bruker ELEXYS E580 spectrometer with X- and Q- (~34.1 GHz) band capabilities was used to collect all pulse EPR spectra. An Oxford Instruments CF935 continuous-flow liquid-helium cryostat was employed for cryogenic temperatures. A Bruker MS5 or MD4 resonator was utilized to collect X-band pulse spectra. A home-built probe based on previously published designs was employed to collect Q-band pulse spectra. All low-temperature data were collected at 10 K with the exception of Davies ENDOR, which was performed at 4.5 K. Additional experimental parameters are listed in the corresponding figure captions. Standard 2-pulse (\(\pi/2-\tau-\pi-\tau\)-echo), 3-pulse (\(\pi/2-\tau-\pi/2-\tau-\pi/2-\tau\)-echo), and 4-pulse (\(\pi/2-\tau-\pi/2-\tau-\tau-\tau\)-echo) electron spin-echo envelope modulation (ESEEM) sequences as well as hyperfine sublevel correlation spectroscopy (HYSCORE, \(\pi/2-\tau-\pi/2-T_1-\tau-T_2-\pi/2-\tau\)-echo) were used to probe the hyperfine coupling between the nitrogen nuclei in close proximity to the Mn(II) ion. Mims (\(\pi/2-\tau-\pi/2-\pi_{RF}-\pi/2-\tau\)-echo) and Davies (\(\pi-\pi_{RF}-\pi/2-\tau-\pi-\tau\)-echo) electron-nuclear double resonance (ENDOR) spectroscopies were used to measure the \(^{15}\text{N}\) and \(^1\text{H}\) hyperfine couplings, respectively. Room temperature X-band EPR (RT-EPR) was performed with 10 \(\mu\text{L}\) of each sample in a capillary melting point tube (Kimble-Chase) using the Bruker SHQE resonator without the cryostat insert.

**EPR Data Analysis.** Time domain spectra were corrected for electron relaxation by subtracting a bi-exponential decay. These time traces were then apodized using a Hamming function and zero-filled (8-fold the length of the original time trace) prior to Fourier-transformation using cross-term averaging (ca. 40-60 cycles) to minimize deadtime-induced artifacts. To determine spin Hamiltonian parameters, simulations were performed using the
The spin Hamiltonian for the $^{55}\text{Mn(II)}$ ($I = 5/2$, 100% natural abundance) spin system is defined by eq. 1.

$$\hat{H} = \beta_e \hat{\mathbf{B}} \cdot \mathbf{g} \cdot \hat{\mathbf{S}} / \hbar + \sum_i (\hat{\mathbf{S}} \cdot \mathbf{A}_i \cdot \hat{\mathbf{I}}_i + \hat{\mathbf{I}}_i \cdot \mathbf{P}_i - \beta_n g_n \beta_n \mathbf{B} \cdot \hat{\mathbf{I}}_i) + \hat{\mathbf{S}} \cdot \mathbf{D} \cdot \hat{\mathbf{S}}$$

The terms, in order, are the electron Zeeman interaction with a static magnetic field $\mathbf{B}$, the electron-nuclear hyperfine (HF) tensor $\mathbf{A}_i$ for every nucleus $i$ with a non-zero nuclear spin ($I$) that is coupled to the Mn(II) center, the nuclear quadrupole tensor $\mathbf{P}_i$ for nuclei with $I > 1/2$, the nuclear Zeeman interaction, and the zero-field splitting (ZFS) tensor $\mathbf{D}$ for the high-spin ($S = 5/2$) Mn(II) ion. $\beta_e$ and $\beta_n$ are the Bohr magneton and nuclear magneton, respectively; $\mathbf{g}$ and $g_n$ are the electron and nuclear $g$ factors, respectively; $\hbar$ is the Planck constant.

The point-dipole approximation (eq. 2) was used to estimate the dipolar contribution to hyperfine tensor anisotropy ($T = [-T, -T, +2T]$ )

$$T_{\text{dip}} = \frac{\mu_0}{4\pi \hbar} \frac{g_e \beta_e g_n \beta_n}{r^3}$$

where $\mu_0$ is the magnetic vacuum permeability and $r$ is the distance between the two magnetic point dipoles, and other parameters are as defined previously.

**Exchangeable Proton Counting.** A published procedure for determining the number of first coordination sphere exchangeable protons was utilized to ascertain the number of water molecules around the Mn(II) center.\textsuperscript{36,37} Three-pulse (3-pulse) ESEEM spectra were collected at a temperature of 10 K using a microwave frequency of 9.5255(5) GHz, and static field strength of 327.35 mT with an 8 ns $\pi/2$ pulse. All 3-pulse ESEEM spectra were collected under identical experimental conditions using protein samples prepared in either H$_2$O or $^2$H$_2$O buffer. In this experiment, a value for $\tau$ was chosen to suppress modulation from protons.\textsuperscript{38} The time domain 3-pulse ESEEM spectrum of Mn(II)-
CP prepared in $^2$H$_2$O buffer was divided by that of Mn(II)-CP prepared in H$_2$O buffer. This quotient spectrum contained modulation only from the hyperfine-coupled solvent-exchangeable deuterons.

To determine the number of bound, inner-sphere exchangeable protons, the contribution to the deuterium modulation from weakly-coupled outer-sphere waters must be removed. Two crystal structures of Mn(II)-bound CP-Ser show that the Mn(II)-binding site in CP is devoid of outer sphere solvent and thus [Mn(DTPA)]$^{3-}$ is an inappropriate standard (vide infra). Thus, we employed the $^2$H$_2$O/H$_2$O ratioed spectrum of Mn(II)-bound CP-Ser (containing 0.9 equivalents Mn(II) and 10 equivalents Ca(II)) as the outer-sphere standard instead of [Mn(DTPA)]$^{3-}$ for all mutants of CP-Ser in order to ascertain whether any change in the hydration level of the Mn(II) ion results from mutation of the Mn(II)-coordinating ligands of the S100A9 C-terminal tail.

Changes in the deuteron modulation depth upon mutation of CP-Ser can be understood in terms of an increase in the number of first-coordination sphere waters or hydroxides by comparison to a standard spectrum of [Mn(H$_2$O)$_6$]$^{2+}$. The modulation depth of [Mn($^2$H$_2$O)$_6$]$^{2+}$/[Mn(H$_2$O)$_6$]$^{2+}$ further divided by the $^2$H$_2$O/H$_2$O ratioed spectrum of [Mn(DTPA)]$^{3-}$ corresponds to six bound water molecules (12 deuterons). Fewer than 12 deuterons are interpolated from the hexaquamanganese spectrum by taking the $^{12}\sqrt{n}$ of the spectrum where n is the number of deuterons.$^{39}$
A4.3 Results and Discussion

X-Ray Crystal Structure of a 1:1 Mn(II)-CP Complex. CP has the ability to exist in a variety of metal-bound forms, making structural elucidation of different CP species necessary for understanding its coordination chemistry and biological function. Despite its propensity to bind Ca(II) and transition metal ions in varying ratios, as well as the significance of M(II)-CP in the immune response, prior to this work only two crystal structures of metal-bound CP were available in the Protein Data Bank (PDB) with accession codes 1XK4 and 4GGF. For this investigation, we sought to obtain a crystal structure of Ca(II)- and Mn(II)-bound CP-Ser in conditions comparable to those employed for pulse EPR spectroscopic characterization to confirm that Mn(II) is coordinated only in the His$_6$ site. On the basis of our initial Mn(II)-binding studies, which revealed that site 2 is the high-affinity Mn(II) binding site ($K_{d, \text{site } 1} = 21 \pm 5 \mu\text{M}$; $K_{d, \text{site } 2} = 194 \pm 203 \text{nM}$; in the presence of 40 equivalents of Ca(II) as ascertained by RT-EPR), we expected that incubation of CP-Ser with 1.0 equivalent of Mn(II) would result in the metal ion only populating the His$_6$ site. We therefore crystallized Ca(II)- and Mn(II)-bound CP-Ser using a 10:1:1 ratio of Ca(II):Mn(II):CP-Ser. The resulting structure is presented in Figure 1. Consistent with our expectations, these conditions afford only one Mn(II) ion bound to each CP-Ser heterodimer. The electron density and anomalous density maps (Figure 1C-E) indicate that Mn(II) is coordinated only by the high-affinity His$_6$ site comprised of His17 and His27 from S100A8 and His91, His95, His103 and His105 from S100A9. The structural attributes of this octahedral Mn(II)-binding site are in excellent agreement with structurally characterized small-molecule Mn(II) hexaimidazole complexes as well as a recently reported structure of Mn(II)-bound CP-Ser (PDB 4GGF) (Tables S4 and S5). In contrast to the 4GGF structure, which revealed Mn(II) bound to the His$_3$Asp site with 50% occupancy in three of the four heterodimers of the
Figure 1. Crystal structure of Ca(II)-, Na(I)-, and Mn(II)-bound CP-Ser (PDB ID 4XJK). (A) The $\alpha_{2}\beta_{2}$ tetramer of CP-Ser. The dashed line represents the dimer-dimer interface. (B) A representation of the $\alpha\beta$ dimer of CP-Ser, taken from the tetramer structure shown in A. (C) Site 1 is a His$_3$Asp motif comprised of (A8)His83, (A8)His87, (A9)His20 and (A9)Asp30. The composite omit electron density map at $3\sigma$ is shown for transition-metal coordinating residues. (D) Site 2 is a His$_6$ motif comprised of residues (A8)His17, (A8)His27, (A9)His91, (A9)His95, (A9)His103, and (A9)His105. The composite omit electron density map at $3\sigma$ is shown for the Mn(II)-coordinating residues and the Mn(II) ion. (E) The anomalous electron density, calculated from data collected at $\lambda = 1.54$ Å, for the His$_6$ site, one canonical, and one non-canonical EF-hand of the S100A8 subunit are contoured at $4\sigma$ with Mn(II), Ca(II), and Na(I) ions, respectively. Additional electron density maps are presented in Figure S4. S100A8 is shown in green, and S100A9 is shown in blue. Transition metal-ion coordinating residues are shown as orange sticks. Ca(II) ions are shown in yellow, Na(I) ions are shown in purple, and Mn(II) ions are shown in magenta.
asymmetric unit, our crystallization conditions result in no electron density corresponding to a bound metal ion at the low-affinity Mn(II) site (Figure 1C). The transition-metal-coordinating residue (A9)His20 is flipped away from the position it needs to be in to coordinate Mn(II) consistent with the CP crystal structure without Mn(II) (PDB 1XK4) (Figure S2). The side chain of (A8)His87 is poorly defined in the electron density map, indicating that the C-terminal region of S100A8 is disordered in the absence of a coordinating transition metal (Figure 1C).

Including 10 equivalents of Ca(II) in the crystallization conditions yielded electron density consistent with Ca(II) bound to each of the two canonical C-terminal EF-hand domains. Initially, we modeled the non-canonical S100A8 and S100A9 N-terminal EF-hands of each heterodimer with Ca(II) ions present with occupancies of 50% and 70%, respectively. In the 1XK4 structure of Ca(II)-bound calprotectin, Ca(II) ions in the non-canonical EF-hand were modeled in at 70% occupancy. In the prior Mn(II)-CP structure (PDB 4GGF), for which stoichiometric Ca(II) was employed in the crystallization conditions, Ca(II) was modeled in the S100A9 non-canonical EF-hand site at 100% occupancy, and the S100A8 non-canonical EF-hand has a water molecule modeled into the site. To further investigate the origins of the electron density in the non-canonical EF-hand domains of the present structure, a dataset collected using our in-house X-ray source was examined. The wavelength at the Cu-K edge (\(\lambda = 1.54 \text{ Å}\)) allows for detection of anomalous signals from both Mn(II) and Ca(II), and the intensity of the manganese anomalous signal is expected to be approximately twice that of the calcium anomalous signal. Using these data, we observe the presence of anomalous density consistent with Mn(II) in the His6 site and Ca(II) in the canonical C-terminal EF-hand domains of each S100 subunit (Figures 1E and S4). In contrast, no anomalous density is observed in the non-canonical EF-hand domains located in the N-terminal regions of S100A8 and S100A9 (Figures 1E and S4). Sodium is present in ca. 1000-fold excess relative to CP-Ser in the protein and crystallization buffers, and Na(I) does not give rise to anomalous scattering. Thus, to best fit the electron density, Na(I) was
modeled into both the S100A8 and S100A9 non-canonical N-terminal EF-hand sites at 100% occupancy. This analysis suggests that excess Na(I) outcompetes Ca(II) at the non-canonical EF-hands under the crystallization conditions. The Ca(II)- and Na(I)-ligand distances for the CP EF-hands are listed in Table S6.

Although the number of metal ions in the three available CP heterotetramer crystal structures differ, the global tertiary structure is unaltered by Mn(II) coordination. The average RMSD between the Cα carbons of the crystal structure of the Ca(II)-bound CP-Ser heterotetramer (PDB 1XK4) and the structure reported in the present work is 0.42 Å, and the variations primarily arise from ordering of the C-terminal tail regions of S100A9 in the presence of Mn(II) (Figure S2). The RMSD between the Cα carbons of the prior Ca(II) - and Mn(II)-bound CP-Ser heterotetramer (PDB 4GGF) and the current structure is 0.21 Å (Figure S3). These comparisons indicate that Mn(II) coordination has a minimal effect on the overall secondary and tertiary structure of CP in the presence of Ca(II).

Figure 2. Hydrogen-bonding interactions at the Mn(II)-His_6 site of Mn(II)- and Ca(II)-bound CP-Ser. Mn(II)-coordinating residues are shown as orange sticks and the Mn(II) ion is represented as a pink sphere. Additional residues are shown as green (S100A8) or blue (S100A9) sticks. The asterisk indicates that the residue is derived from an adjacent “dimer” of the α2β2 tetramer.
Our refinement indicates that the Mn(II)-His$_6$ site excludes solvent water from the primary coordination sphere, resulting in a remarkably dry Mn(II) coordination environment. Furthermore, there are hydrogen bonding interactions between the Mn(II)-coordinating His residues and additional protein side chains. We hypothesize that these interactions limit water in the secondary coordination sphere. Indeed, only two ordered water molecules are observed in the secondary coordination sphere of Mn(II) at site 2 (Figure 2). The atoms (A9)His91 Nδ1 and (A9)His103 Nδ1 each form a hydrogen-bonding interaction with these ordered solvent water molecules. Additionally, (A8)His17 Nδ1 forms a hydrogen bond with the backbone carbonyl of (A8)Ile13, and (A9)His95 Nδ1 forms a hydrogen bond with (A9)Asp98. The atom (A8)His27 Nδ1 forms a hydrogen bond at the dimer-dimer interface with Asp65 from the second S100A9 polypeptide chain (Figure 2). These hydrogen-bonding interactions may contribute to the thermodynamic stability of Mn(II)- and Ca(II)-bound CP as well as charge neutralization at the His$_6$ site.

**Table 2. Zero-field splitting parameters of select six-coordinate Mn(II) complexes**

| Complex/Protein       | $|D|$ (MHz) | $|E|$ (MHz) | $E/D$ | Coordination Motif | Reference |
|-----------------------|------------|------------|-------|--------------------|-----------|
| Mn(Im)$_6$ $^a$       | 354        | 66         | 0.19  | N$_6$              | 46        |
| CP-Ser                | 270        | 81         | 0.30  | N$_6$              | 10        |
| CP-Ser                | 485        | 145.5      | 0.30  | N$_6$              | This work |
| ΔHis$_3$Asp           | 485        | 145.5      | 0.30  | N$_6$              | This work |
| H103A                 | 650        | 105        | 0.30  | N$_5$O$_1$         | This work |
| OxDc (site 1) $^b$    | 1200       | 250        | 0.21  | N$_3$O$_3$         | 47        |
| OxDc (site 2)         | 2700       | 675        | 0.25  | N$_3$O$_3$         | 47        |
| Concanavalin A        | 645        | 71         | 0.11  | N$_3$O$_5$         | 48        |
| Creatine kinase (PO$_4^{3-}$) $^c$ | 673 | 108 | 0.16 | O$_6$ | 49 |
| Creatine kinase (NO$_3^-$) $^c$ | 1471 | 191 | 0.13 | O$_6$ | 49 |
| Creatine kinase (NO$_2^-$) $^c$ | 1121 | 370 | 0.33 | O$_6$ | 49 |
| FosA + fosfomycin $^d$ | 6900 | 600 | 0.09 | N$_2$O$_3$ | 50 |

$^a$Mn(II) hexaimidazole. $^b$ Oxalate decarboxylase. $^c$ Variable anions that prevent reactivity are included in the creatine kinase simulations. All reported numbers are the absolute value. $^d$ Fosfomycin is the substrate of FosA.
Mn(II) X-Band CW EPR of CP-Ser and Mutant Proteins in the Presence of Ca(II).

The CW EPR spectrum of Ca(II)- and Mn(II)-bound CP-Ser (75 mM HEPES, 100 mM NaCl, pH 7.5, 10 equiv. of Ca(II)) was previously reported, and the Spincount simulation ($D = 270$ MHz, $E/D = 0.30$, Table 2) indicated a nearly idealized octahedral Mn(II) coordination sphere. In the current work, we further probed the Mn(II) coordination sphere of CP-Ser and select metal-binding site mutants using CW and pulse EPR spectroscopies. The EPR samples were prepared with 0.9 equivalents of Mn(II) and 10 equivalents of Ca(II). Under these conditions, we expect that Mn(II) is bound only at the His$_6$ site and all four EF-hand domains contain Ca(II) or Na(I), as seen in the crystal structure presented in Figure 1. Representative low temperature (10 K) X-band (9.4 GHz) CW EPR spectra of CP-Ser, and the AHis$_3$ Asp and H103A mutants are presented in Figure 3. Additional spectra of CP mutants are presented in Figure S5.

The isotropic $g$-value of 2.001 observed for these samples is typical for high-spin $d^6$ Mn(II) ions and results from the near-spherical symmetry of the electron spin distribution around the Mn(II) center. The sextet of EPR features centered at $g = 2.001$ arises from the hyperfine coupling of the $^{55}$Mn nucleus ($I = 5/2$, 100% abundance) with the $m_s = \pm 1/2$ electron spin manifolds. The hyperfine-induced splitting of the four outer manifold ($m_s = \pm 1/2$ $\leftrightarrow m_s = \pm 3/2$ and $m_s = \pm 3/2$ $\leftrightarrow m_s = \pm 5/2$) EPR transitions is rarely resolved in spectra of frozen solutions because of the orientation-dependent spectral broadening of EPR transitions in these spin manifolds. The spectra in Figure 3 reveal that both CP-Ser and AHis$_3$ Asp samples exhibit identical Mn(II) spectroscopic signatures. This result is in agreement with prior work and indicates that there is no Mn(II) coordinated by site 1 under these conditions. For CP-Ser and the AHis$_3$ Asp mutant, the hyperfine coupling to the $^{55}$Mn nucleus was found to be isotropic with a magnitude of 247 MHz. This value is low relative to published values; reported $^{55}$Mn hyperfine coupling values are typically $> 255$ MHz. The low coupling constant is consistent with the high number of His ligands and the reported trend of decreased $^{55}$Mn hyperfine coupling as more imidazoles are bound to a Mn(II) ion.
Between each pair of lines of the sextet lie two formally forbidden $\Delta m_s = \pm 1$, $\Delta m_I = \pm 1$ transitions. By paying particular attention to the ratio of the intensity of the forbidden EPR transitions to that of the allowed EPR transitions, as well as the overall spectral lineshape and width, simulation of the CP-Ser spectrum (Figure 3A) using EasySpin afforded a value for the axial component of the zero-field splitting tensor, $D = 485$ MHz ($E/D = 0.30$). Although this $D$ value is somewhat larger than the $D$ value obtained previously, it is still small compared to the reported $D$ values for other Mn(II) complexes (Table 2). A low $D$ value can be indicative of a highly symmetric coordination environment, in accordance with the hexahistidine Mn(II)-binding site observed crystallographically. The $\Delta$His$_3$Asp spectrum can be simulated using the same parameters as CP-Ser (Figure 3B), which provides further support for the notion that Mn(II) is exclusively coordinated by the His$_6$ motif under these conditions.

Figure 3. X-band CW EPR spectra (black traces) and simulations (gray traces) of Mn(II)- and Ca(II)-bound CP-Ser (A), $\Delta$His$_3$Asp (B), and H103A (C). Simulation parameters are given in Table 2. Spectrometer settings: $\nu_{\text{mw}} = 9.4$ GHz, 0.5 mT modulation at 100 KHz, power = 0.2 mW, temperature = 10 K. Sample conditions: 200 $\mu$M CP, 180 $\mu$M Mn(II), 2.0 mM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).
The spectrum of the Mn(II)-H103A mutant reveals that, upon mutation of one of the Mn(II)-coordinating His residues to Ala, the intensity of the forbidden transitions relative to the allowed transitions increases significantly (Figure 3C). This increase in forbidden transition intensity was also seen in the spectra of CP upon single point mutations of the His4 motif. Such forbidden transitions typically gain intensity as the ZFS increases. In agreement with this observation, simulation of the Mn(II)-H103A signal afforded a $D$ value of 650 MHz (Figure 3C). This $D$ value remains in the range for six-coordinate Mn(II) ($|D| < 3000$ MHz), but suggests that the symmetry of the Mn(II)-binding site has decreased as expected with the loss of one histidine ligand.

**Q-Band ENDOR and ESEEM Spectroscopic Studies Confirm a Highly Symmetric Mn(II)-His$_6$ Site in Solution in the Presence of Ca(II).** Pulse EPR spectroscopy was employed to further characterize the nitrogen atoms of the high-affinity Mn(II)-His$_6$ site in the presence of 10 equivalents of Ca(II). Samples of CP-Ser were globally labeled with $^{15}$N ($I = 1/2$)—denoted $^{15}$N-CP-Ser—in order to probe only the hyperfine interaction by eliminating the quadrupole interaction intrinsic to the $^{14}$N ($I = 1, 99.6\%$ abundance) nucleus. The Q-band (≈34.1 GHz) Mims-ENDOR spectrum (Figure 4) of Mn(II)- and Ca(II)-bound $^{15}$N-CP-Ser revealed two sets of doublets centered at the $^{15}$N Larmor frequency (5.3 MHz at 1222.5 mT). The full hyperfine coupling tensors for the two classes of $^{15}$N nuclei are summarized in Table 3. We assign the broader, larger spaced doublet (denoted A in Figure 4), simulated with $a_{iso} = 4.36$ MHz, to $^{15}$N of the proximal histidine nitrogens (Nε2) that directly coordinate Mn(II). These peaks are artificially split by tau-dependent blind spots intrinsic to Mims-ENDOR (so-called Mims holes), an effect that is verified by collecting the data at different tau values (Figure S6). The narrower, more closely spaced doublet (denoted B) is simulated with the isotropic hyperfine coupling ($a_{iso}$) of 0.24 MHz, and is assigned to the distal nitrogen atoms (Nδ1) on the imidazole rings. The approximately 18x larger coupling of Nε2 relative to Nδ1 is consistent with the ratio of
proximal-to-distal nitrogen isotropic hyperfine couplings observed in other histidine-coordinated metal systems.\textsuperscript{57-59} The lack of more than two sets of doublets in the \textsuperscript{15}N ENDOR spectrum demonstrates the spectroscopic equivalence of the histidine moieties that comprise the Mn(II)-binding site. These couplings are to the central $m_S = \pm 1/2$ manifolds of the $S = 5/2$ Mn(II) ion. Couplings to the higher $m_S$ manifolds are weak (see * in Figure 4) and are not analyzed in this work.

The Q-band time domain 3-pulse and 4-pulse ESEEM spectra of \textsuperscript{15}N-CP-Ser (+Ca) are presented in Figure 5A. These \textsuperscript{15}N ESEEM spectra are well simulated using the coupling constants derived from the above ENDOR spectroscopic study (Figure 4). The corresponding Fourier transform of the 4-pulse ESEEM data is presented in Figure 5B. The \textsuperscript{15}N transitions of the coordinating imidazole nitrogen atoms are the pair of features centered at 3.1 and 7.5 MHz. The sharp peak at 10.6 MHz results from the sum of these two fundamental \textsuperscript{15}N frequencies. This combination peak is naturally sharper than the fundamental peaks because orientation dependences in each fundamental peak are nulled in the sum combination peak.\textsuperscript{60} The modulation in the 4-pulse ESEEM spectrum is remarkably long-lived (>19 \textmu sec) and indicative of well-defined and equivalent \textsuperscript{15}N frequencies from the six coordinating NE2 ligands.
Figure 4. Mims ENDOR spectrum (black) of Mn(II)- and Ca(II)-bound $^{15}$N-CP-Ser collected at $\tau = 500$ ns and simulation (gray). A corresponds to the proximal nitrogen of the imidazole ring. B corresponds to the distal nitrogen of the imidazole ring. The asterisk (*) indicates $^{15}$N ENDOR transitions coupled to the $m_\sigma = 1/2 \leftrightarrow m_\sigma = 3/2$ EPR transitions. Simulation of $m_\sigma = \pm 1/2$ for the two classes of nitrogen atoms (gray) were obtained using the parameters listed in Table 3. Spectra at additional $\tau$ values are presented in Figure S6. Spectrometer settings: $\nu_{mw} = 34.1$ GHz, $B_0 = 1222.5$ mT, 8 ns $\pi/2$, temperature = 10 K. Sample conditions: 200 $\mu$M $^{15}$N-CP-Ser, 180 $\mu$M Mn(II), 2.0 mM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).

Table 3. Hyperfine coupling (MHz) to magnetic nuclei in CP-Ser $^a$

<table>
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<tr>
<th>Nucleus</th>
<th>$a_{iso}$</th>
<th>$a_1$</th>
<th>$a_2$</th>
<th>$a_3$</th>
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<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
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<td>$^{15}$Nε2 (proximal)</td>
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<tr>
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<td>2.64</td>
<td>4.21</td>
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<td>$^{14}$Nδ1 (distal)</td>
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<td>0.08</td>
<td>0.13</td>
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<tr>
<td>Mn(Im$_6$) (proximal $^{14}$N)</td>
<td>3.19</td>
<td>2.50</td>
<td>2.62</td>
<td>4.45</td>
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</tbody>
</table>

$^a$ Samples contained 200 $\mu$M CP-Ser at pH 7.5 (75 mM HEPES, 100 mM NaCl) with 10 equivalents of Ca(II) and 0.9 equivalents of Mn(II). $^b$ n.a., not applicable. $^c$ Mn(Im)$_6$ hyperfine coupling constants are taken from reference $^{46}$. 

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Figure 5. 3-pulse (purple line) and 4-pulse (red line) ESEEM of Mn(II)- and Ca(II)-bound \(^{15}\)N-labeled CP-Ser at 34.1 GHz. The top panel (A) is the time domain. The bottom panel (B) is the Fourier transform of the 4-pulse ESEEM time domain. The black traces represent simulations of each spectrum, and the parameters are listed in Table 3. Spectrometer settings: \(v_{\text{mw}} = 34.1\) GHz, \(\pi/2 = 8\) ns, \(\tau = 200\) ns, \(T_0 = 120\) ns, temperature = 10 K, shot repetition time = 1500 \(\mu s\), \(B_0 = 1223.7\) mT. Sample conditions: 200 \(\mu M\) \(^{15}\)N-CP-Ser, 180 \(\mu M\) Mn(II), 2.0 mM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20\% (v/v) PEG 200).

In addition to globally labeled \(^{15}\)N-CP-Ser, we prepared mixed-labeled samples of \(\Delta\text{His}_3\text{Asp}\) where only one subunit was \(^{15}\)N-labeled. We accomplished this labeling scheme by expressing one S100A8/S100A9 subunit in minimal medium containing \(^{15}\)N ammonium chloride as the sole nitrogen source and the other subunit in minimal medium with nitrogen at natural abundance. This procedure provided a set of samples with zero \(^{15}\)N (a natural abundance sample), two \(^{15}\)N (\(^{15}\)N(A8)-\(\Delta\text{His}_3\text{Asp}\)), four \(^{15}\)N (\(^{15}\)N(A9)-\(\Delta\text{His}_3\text{Asp}\)), and six \(^{15}\)N (\(^{15}\)N-\(\Delta\text{His}_3\text{Asp}\)) derived from \(^{15}\)N-labeled His residues contributing to the Mn(II) coordination sphere. The 4-pulse ESEEM Fourier transformation for each of these four samples are
shown in Figure 6. The inset shows an approximately linear trend in the amplitude of the combination $^{15}$N peak at 10.6 MHz with respect to the number of $^{15}$N nitrogen ligands. Hyperfine sublevel correlation (HYSCORE) spectra of fully and partially $^{15}$N-labeled species are presented in Figures S7-S10.

![Figure 6](image)

Figure 6. Frequency domain of 4-pulse ESEEM spectra of Mn(II)- and Ca(II)-bound mixed $^{14}$N/$^{15}$N-labeled ΔHis$_3$Asp with 0.9 equivalents of Mn(II). The inset is a graph of maximum intensity of the combination peak vs the number of $^{15}$N ligands. The black * represents the intensity of the $^{15}$N sum combination line measured for $^{15}$N-labeled Mn(II)-CP in the absence of Ca(II) (vide infra) and was not used in the calculation of the best fit line. The number of $^{15}$N ligands for this point was calculated using the linear relationship determined from the best-fit line. Spectrometer settings: $\nu_{mw} = 34.1$ GHz, $B_0 = 1240$ mT, $\pi/2 = 8$ ns, $\tau = 200$ ns, $T_0 = 40$ ns, temperature = 10 K. All samples contained 200 μM ΔHis$_3$Asp, 2 mM Ca(II), and 180 μM Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).

Exchangeable Proton Counting Reveals that the C-Terminal Tail of S100A9 Prevents Water Access to Bound Mn(II). The amino acid sequence of the C-terminal tail, defined as residues 96-114 of S100A9, is EGDEGPGHHKPKGEGTP where the Mn(II)-coordinating His103 and His105 residues are shown in boldface. The crystal structure of the Ca(II)-bound CP-Ser heterotetramer (PDB 1XK4) revealed a disordered tail region, indicating that the tail is dynamic in the absence of Mn(II). We previously characterized the
Mn(II)-binding properties and antimicrobial activity of a mutant family to probe the consequences of perturbation of the HXH motif on CP function, and hypothesized that solvent water may contribute to Mn(II) coordination in the absence of His103 and/or His105. To evaluate this notion, we employed 3-pulse ESEEM spectroscopy to determine the number of exchangeable deuterons in the immediate vicinity of the Mn(II) ion bound to H103A, H104A, H105A, AHA, and AAA mutants of CP in the presence of 10-fold Ca(II) (see the Experimental section for details on this procedure). In previous water-counting studies using 2H2O-enriched buffer, we employed [Mn(DTPA)]3- (DTPA = diethylaminetriaminepentaacetic acid) as a spectroscopic standard for Mn(II) with zero bound water because the hexacoordinate DTPA chelating ligand excludes water from the first coordination sphere of manganese; however, the outer sphere is fully exposed to solvent 2H2O. Figure 7 compares the 2H2O/H2O ratioed 3-pulse ESEEM spectra, which eliminates contributions from other magnetic nuclei such as 14N, of Mn(II)-bound CP and [Mn(DTPA)]3-. The 2H modulation of Mn(II)-CP is appreciably weaker than that of the DTPA chelate, demonstrating that the Mn(II) has no inner sphere waters and that its hexahistidine site is shielded from 2H exchange into the outer sphere as well. A simulation using a dipolar coupling of 0.26 MHz (r = 3.6 Å) partially reproduces the persistent 2H modulation in the 2H2O/1H2O ratioed 3-pulse ESEEM spectrum of CP-Ser. Further evidence for the absence of inner-sphere exchangeable deuterons in CP-Ser is provided by the proton Davies ENDOR spectra of Mn(II)- and Ca(II)-bound CP-Ser in H2O and 2H2O buffer (Figure S11). A comparison of these spectra reveals that exchanging Mn(II)- and Ca(II)-bound CP-Ser into 2H2O buffer has only a modest effect on the 1H ENDOR spectrum, decreasing the intensity only at the 1H Larmor frequency, which indicates that only weakly coupled and distant protons are replaced. Thus, the pulse EPR results of Mn(II)- and Ca(II)-bound CP-Ser in solution are consistent with the dry Mn(II) environment revealed in our X-ray structure, which was obtained at comparable Mn(II) and Ca(II) concentrations (Figure 2).
We next examined the effect of mutating histidine ligands of the S100A9 tail region on the hydration of the Mn(II) center. The double ratioed \( ^2\text{H}_2\text{O/}^2\text{H}_2\text{O [mutant]}/^2\text{H}_2\text{O/}^2\text{H}_2\text{O [CP-Ser]} \) ESEEM spectra are shown in Figure 8. This presentation emphasizes the changes induced by the specific mutation. Standard modulation curves for 1 through 4 inner sphere deuterons are provided (see Experimental Section). H104 does not coordinate Mn(II) and, as expected, the H104A mutant shows no additional deuteron modulation relative to the inner sphere deuteron standards. Mutations at the two tail histidine ligand sites introduce appreciable \( ^2\text{H} \) modulation, with approximately one inner sphere deuteron appearing in the H105A mutant and close to two.
Figure 8. Exchangeable proton counting of Mn(II) bound to H103A, H104A, H105A, AHA, or AAA. Black lines are calculated modulations for 1, 2, or 3 coordinating deuterons. Spectrometer settings: $\nu_{mw} = 9.525(5)$ GHz, $B_0 = 327.35$ mT, $8 \text{ ns } \pi/2$, $\tau = 216$ ns, $T_0 = 80$ ns, temperature = 10 K. Sample conditions: 200 $\mu$M CP, 180 $\mu$M Mn(II), 2.0 mM Ca(II), pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).

inner sphere deuterons appearing in the H103A mutant. Furthermore, the AHA and AAA mutants, both of which lack the Mn(II)-coordinating His residues of the tail, show deep deuteron modulation, consistent with three inner sphere deuterons. Compared to the shallow and persistent $^2$H modulation of CP-Ser, the inner sphere $^2$H modulation damps quickly in the time domain, corresponding to wider peaks in the Fourier transform (not
shown) due to the stronger dipolar hyperfine couplings. We conclude that in the absence of the histidine ligands from the tail, water and/or hydroxide can access the Mn(II) inner coordination sphere.

A decrease in symmetry of the Mn(II) center will result from loss of a native histidine ligand and occupation of the vacant Mn(II) coordination site with a water molecule or hydroxide ion. Indeed, the CW EPR spectra of the H103A and H105A mutants (Figures 3 and S4) indicate that the symmetry of the Mn(II)-binding site has been dramatically lowered with loss of a histidine ligand, as supported by a significant increase in the magnitude of the ZFS interaction. Simulation of the CW EPR data for the H103A mutant yielded a $D$ value of approximately 650 MHz (Table 2). For the spectrum of the H105A mutant, a value of $D$ could not be determined as a result of the more complex line shape; however, the ratio of the allowed EPR transition intensity to that of the forbidden transitions is diminished compared to that observed in the spectrum of the H103A mutant, which indicates that the Mn(II) bound to H105A CP-Ser (Figure S4) has the highest ZFS of the single point mutants we investigated (> 650 MHz). Thus, based on the ZFS parameters discerned from analysis of the CW EPR spectra of the CP mutants, the Mn(II) ion coordination environment becomes less symmetric according to the trend CP-Ser > H103A > H105A with the H105A mutant exhibiting the lowest symmetry.

The ESEEM and CW EPR data together establish that the Mn(II) ion at the His$_6$ site becomes solvent exposed in the absence of His103 and/or His105. The direct demonstration of increased solvent accessibility upon mutating residues of the His$_6$ site is important for understanding the ability of CP to sequester labile Mn(II). Our results are consistent with a model where encapsulation of Mn(II) by the C-terminal tail of S100A9 is essential for metal sequestration, and that solvent exposure makes the Mn(II) ion more kinetically labile. Although the AHA and AAA mutants retain the ability to bind Mn(II), these mutants are unable to sequester Mn(II).
We note that capture of Mn(II) at the His₆ site of Ca(II)-bound CP is reminiscent of synthetic small-molecule cages designed to coordinate first-row transition metal ions with high affinity. In particular, the sarcophagines are macrobicyclic hexa-amine ligands that encapsulate labile metal ions, including high-spin Mn(II).[^2][^3]

**Mn(II) Binding to CP-Ser is Promiscuous in the Absence of Ca(II).** Because the Mn(II)-chelating properties of CP are Ca(II)-dependent, we next applied EPR to further characterize Mn(II)-CP in the absence of Ca(II) with the goal of ascertaining whether changes in the Mn(II) electronic/geometric structure or speciation occur. In prior work, we concluded that CP-Ser (-Ca) binds Mn(II) with micromolar affinity at site 2 ($K_{d,\,site \,2} = 4.9 \pm 1.0 \, \mu M$, determined by RT-EPR titrations).[^10] These studies also demonstrated that the interfacial His₄ motif of site 2 is required for appreciable Mn(II) complexation in the absence of Ca(II).[^10] On the basis of the observed micromolar Mn(II) affinity of CP-Ser (-Ca), and to minimize free Mn(II) in the sample, we prepared a Mn(II):CP sample with 180 μM Mn(II) and 1.0 mM CP-Ser (*vide infra*).
Figure 9. X-band CW EPR spectra of (A) Mn(II)-bound CP-Ser in H₂O, (B) Mn(II)-bound CP-Ser in ²H₂O, (C) Mn(II)- and Ca(II)-bound CP-Ser in ²H₂O, (D) 200 μM MnCl₂ in ²H₂O with 20% (v/v) PEG 200. The gray trace is a linear combination of ½ C and ½ D. Spectrometer settings: \( \nu_mw = 9.4 \text{ GHz}, 0.5 \text{ mT modulation at 100 KHz}, \) power = 0.2 mW, temperature = 10 K. Protein sample conditions: 1 mM CP-Ser (−Ca) or 200 μM CP-Ser (+Ca), 180 μM Mn(II), pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200), ± 2 mM Ca(II).

In agreement with our prior work, the low-temperature X-band CW EPR spectra of Mn(II)-CP-Ser in the presence and absence of excess Ca(II) markedly differ (Figures 3A and 9A). In the absence of Ca(II), all of the spectral features for Mn(II) appear to broaden significantly. When the –Ca(II) sample was buffer exchanged into buffer prepared with ²H₂O, sharpening of some features occurred (traces A and B in Figure 9). This observation is consistent with significant hyperfine interactions between the Mn(II) and solvent-exchangeable hydrogen. Moreover, in the spectrum of the –Ca(II) sample prepared in ²H₂O, the lowest-field member of the Mn(II) sextet centered at \(-25 \text{ mT}\) is clearly split (Figure 9B), which suggests that at least two magnetically distinct Mn(II)-containing species contribute to the EPR spectrum in this region. This splitting can be approximately modeled by a linear...
combination of the \(^2\text{H}_2\text{O}\)-exchanged Mn(II)- and Ca(II)-bound CP-Ser spectrum (Figure 9C) and a spectrum of \([\text{Mn}(^2\text{H}_2\text{O})_6]^{2+}\) (Figure 9D) in a 1:1 ratio (Figure 9B, gray trace).

In order to further evaluate the Mn(II) speciation in the −Ca(II) sample, room-temperature EPR (RT-EPR) was employed to quantify the amount of unbound Mn(II) in the sample. Whereas \([\text{Mn(H}_2\text{O})]^{2+}\) gives a six-line pattern centered at \(g = 2\) at room temperature, Mn(II) coordinated by macromolecules does not give rise to a detectable signal. Furthermore, the RT-EPR Mn(II) signal is quantitative and allows for the concentration of unbound Mn(II) to be determined. Analysis of the 0.18:1.0 Mn(II):CP-Ser prepared in the absence of Ca(II) revealed a RT-EPR signal that accounted for ca. 10-20% (20-40 \(\mu\text{M}\)) of the total Mn(II) (Figure S12). We note that the observed quantity of free Mn(II) is greater than the expected amount calculated by using a \(K_d\) value of \(\approx 5 \, \mu\text{M}\) that was determined for CP-Ser (−Ca) by RT-EPR. The amount of free Mn(II) concentration increased to approximately 50% of the total when 0.9 equiv of Mn(II) was added to CP-Ser in the absence of Ca(II) (Figure S12). On the basis of this quantification, and to minimize the free Mn(II) signal, we decided to further investigate Mn(II) complexation by CP-Ser in the absence of added Ca(II) using samples prepared with 0.18:1 Mn(II):CP-Ser.

In prior Mn(II)-binding studies of CP-Ser, the inclusion of additional low-affinity (1.0 mM) Mn(II)-binding events was necessary to fit the RT-EPR Mn(II) titration curve. This analysis indicated the presence of additional Mn(II)-binding sites in the absence of Ca(II). To investigate this possibility, the speciation of Mn(II)-CP (−Ca) was interrogated by pulse EPR. The 4-pulse ESEEM Fourier transforms of \(^{15}\text{N}\)-CP-Ser in the presence and absence of Ca(II) are compared in Figure 10. The lineshapes of the \(^{15}\text{N}\) ESEEM and ENDOR (Figures 10 and 11, respectively) are the same for −Ca and +Ca samples, showing there is no new or different nitrogen coordination to Mn(II) in the absence of Ca(II). Nevertheless, the \(^{15}\text{N}\) 4-pulse ESEEM combination peak amplitude of Mn(II)-CP (−Ca) corresponds to only ca. 2.6 nitrogens per Mn(II) (Figure 6 inset). This observation supports our analysis of the low-temperature CW EPR spectra (Figure 9) in that ca. 50% of the Mn(II) is coordinated by the
His$_6$ site in the absence of Ca(II) in this sample containing a 0.18:1 Mn(II):CP-Ser molar ratio. An analysis of the natural abundance $^{14}$N modulation in 3-pulse ESEEM data also supports this conclusion (*vide infra*).

![Figure 10](image)

**Figure 10.** 4-pulse ESEEM frequency domain of 0.18:1 Mn(II):$^{15}$N-CP-Ser (red) and Mn(II)- and Ca(II)-bound to $^{15}$N-CP-Ser. The concentration of Mn(II) in both spectra is 180 μM. Spectrometer settings: $v_{mw}$ = 34.1 GHz, $B_0$ ~ 1240 mT, $\pi/2$ = 8 ns, $\tau$ = 200 ns, $T_0$ = 80 ns, temperature = 10 K. Protein sample conditions: 1 mM CP-Ser (−Ca) or 200 μM CP-Ser (+Ca), 180 μM Mn(II), pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200, ± 2 mM Ca(II)).
Figure 11. Mims-ENDOR of Mn(II)-bound $^{15}\text{N}$-CP-Ser in the absence (red) and presence (green) of Ca(II). Spectrometer settings: $\nu_{mw} = 34$ GHz, $B_0 \sim 1240$ mT, $\pi/2 = 8$ ns, $\tau = 448$ ns, $T = 400$ ns, temperature = 10 K. Protein sample conditions: 1 mM CP-Ser (−Ca) or 200 μM CP-Ser (+Ca), 180 μM Mn(II), pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200), ± 2 mM Ca(II).

Next, the degree of hydration of the Mn(II) site(s) in the absence of Ca(II) was assessed by ESEEM as described for the +Ca(II) samples. Consistent with prior observations, the hydration level increases with increasing Mn(II) in the −Ca(II) samples. Indeed, the samples prepared with 0.18:1.0 and 0.9:1.0 Mn(II):CP-Ser ratios have approximately one and two exchangeable protons, respectively (Figure 12). The $^2\text{H}_2\text{O}/\text{H}_2\text{O}$ ratioed 3-pulse ESEEM, spectrum of CP-Ser (−Ca) with 0.18 equivalents of Mn(II) shows a significant increase in the amount of residual $^2\text{H}$ modulation compared to that for the corresponding +Ca(II) sample (Figure 13). This deuterium modulation can be accounted for by scaling the $^2\text{H}_2\text{O}/\text{H}_2\text{O}$ ratioed 3-pulse ESEEM spectrum of aqueous Mn(II) to correspond to 12% of the total Mn(II) concentration. This value is within the 10-20% range of unbound Mn(II) predicted by our RT-EPR analysis (Figure S12). Thus, all inner-sphere water detected
in the ratioed ESEEM spectrum can be accounted for by the fraction of aqueous Mn(II) in the –Ca sample.

![Graph](image)

**Figure 12.** Exchangeable proton counting of Mn(II)-bound CP-Ser with varying ratios of Mn / CP. Spectrometer settings: $v_{mw} = 9.525(5)$ GHz, $B_0 = 327.35$ mT, $\pi/2 = 8$ ns, $\tau = 216$ ns, $T_0 = 80$ ns, temperature = 10 K. Protein sample conditions: 1 mM CP-Ser (red) or 200 µM CP-Ser (blue), 180 µM Mn(II), pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).

In total, these data suggest that Mn(II) binds to several different sites in CP when Ca(II) is omitted from the buffer. For a 1.0 mM CP-Ser (–Ca) sample containing 180 µM Mn(II), we determine that ≈90 µM of the Mn(II) is coordinated by the His$_6$ site and 20-40 µM exists as aqueous Mn(II). Because no new features are observed in either the $^{15}$N 4-pulse ESEEM or the Mims ENDOR spectra (Figures 10 and 11) as a result of omitting Ca(II) from the Mn(II)-CP sample, the remainder of Mn(II) (≈50-70 µM) must be bound to a site (or sites) that do not contain nitrogen ligands. Furthermore, the additional Mn(II)-binding site(s) appears to coordinatively saturate the Mn(II) ion as there is no $^2$H modulation in the
The "H_2O/H_2O ratioed 3-pulse ESEEM spectrum of the -Ca(II) sample that cannot be attributed to the modest amount of aqueous Mn(II) observed by RT-EPR spectroscopy.

Figure 13. "H_2O/H_2O 3-pulse ESEEM time domain of Mn(II)-bound CP-Ser in the absence (red) and presence (green) of Ca(II). The protein samples contain 180 μM of Mn(II), and an equivalent concentration of manganese hexaqua is shown in black. The gray line overlaying the -Ca(II) CP-Ser data is a linear combination of 0.12*hexaquamanganese and 0.88*Mn(II) and Ca(II)-bound CP-Ser. Spectrometer settings: \( v_{mw} = 9.525(5) \) GHz, \( B_0 = 327.35 \) mT, \( \tau/2 = 8 \) ns, \( T = 216 \) ns, \( T_0 = 80 \) ns, temperature = 10 K. Sample conditions: 1.0 mM CP-Ser (-Ca) or 200 μM CP-Ser (+Ca), 180 μM Mn(II), pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200), ± 2 mM Ca(II).

Candidates for the additional Mn(II) sites include site 1 (His3Asp) and the EF-hand domains. The previous Mn(II)-bound crystal structure (PDB ID 4GGF), obtained with Ca(II) present in the crystallization conditions, showed Mn(II) bound at Site 1 in a 5-coordinate geometry with a bidentate interaction from Asp30.13 Pentacoordinate Mn(II) sites are characterized by large zero-field splittings (\( D \) is typically greater than 3000 MHz) and give rise to distinct EPR spectra.50,66,67 We see no such evidence for such spectroscopic features in the CW EPR spectrum of the -Ca(II) sample. Nevertheless, simulations of Mn(II) EPR
spectra with ZFS values ranging from 1,000 to 10,000 MHz, along with a representative Mn(II) spectrum with a ZFS of 500 MHz indicate that detecting a high-ZFS species in the presence of the Mn(II)-His$_6$ signal is difficult (Figure S14).

The EF-hand domains provide multi-dentate coordination spheres comprised of oxygen donor atoms (Table 1), and select EF-hand domains are reported to chelate Mn(II)$^{68,69}$ For instance, a crystal structure of the N-terminal domain of calmodulin crystallized in the presence of Mn(II) revealed an octahedral Mn(II) center where the primary coordination sphere includes two solvent water molecules.$^{69}$ In prior work, we suggested that Mn(II) may associate with the vacant EF-hand domains of CP-Ser with low affinity$^1$ and our current investigation provides additional support for Mn(II) binding in such coordination environments. Indeed, the ligand framework provided by an EF-hand domain is expected to give rise to a Mn(II) CW EPR spectrum with similar $g$ and $A$ values as $[\text{Mn(H}_2\text{O)}_6]^{2+}$.$^{47,48,68}$ Although Mn(II) binding in the His$_3$Asp site remains a possibility on the basis of the current spectroscopic results, we propose that in the absence of Ca(II), Mn(II) is coordinated by the His$_6$ site as well as the EF-hand domains of CP-Ser. In prior studies, we observed no Mn(II) binding to CP-Ser-$\Delta$His$_4$ (site 2 mutant) or $\Delta$ (site 1 / site 2 mutant) by RT-EPR in the absence of Ca(II).$^{10}$ Thus, we reason that the EF-hand domains cannot bind Mn(II) to an appreciable degree in the absence of site 2. Taken together with our current proposal, it appears that Mn(II) chelation at site 2 enhances the Mn(II) affinity of the EF-hands, which suggests cooperativity between the His$_6$ site and EF-hand domains. Each CP heterodimer unit harbors four different EF-hand domains, and elucidating the identities of the putative Mn(II)-binding EF-hands is a subject for future work. On the basis of prior studies$^{68,69}$ and the X-ray crystallographic work presented here, we reason that the C-terminal canonical EF-hands of S100A8 and S100A9 are the most probable sites for Mn(II) binding.
Summary

In the current work, we address the Ca(II)-dependent Mn(II)-binding properties of the human host-defense protein CP, and present crystallographic and EPR spectroscopic characterization of human CP in the presence of Mn(II) and Ca(II). In support of our prior solution studies,\textsuperscript{10} the crystal structure presented here confirms that Mn(II) is coordinated solely at the His\textsubscript{6} site when one equivalent of Mn(II) is present. Anomalous diffraction data establishes that the canonical C-terminal EF-hands coordinate Ca(II) whereas the non-canonical N-terminal EF-hands are populated by another ion, reasoned to be Na(I), under crystallographic conditions that included 10 equivalents of Ca(II). This result is consistent with the low Ca(II) affinities reported for characterized non-canonical EF-hands,\textsuperscript{20,22,24,25} and further structural and biochemical investigations of CP to evaluate the interplay between Ca(II) and Na(I) are warranted. We note that S100A2 and S100A12 have also been crystallized and refined with Na(I) in the non-canonical EF-hands.\textsuperscript{70,71}

The application of EPR spectroscopic methods to studies of CP in the presence of Ca(II) establishes several important facets about how CP sequesters Mn(II) at the His\textsubscript{6} site. Mn(II) is bound to the His\textsubscript{6} site with a high degree of electronic symmetry as evidenced by the low $D$ value (Table 2) and in agreement with the symmetric His\textsubscript{6} site observed crystallographically. Exchangeable proton counting confirmed that mutation of the S100A9 C-terminal tail perturbs the Mn(II) coordination environment and enables solvent access to the Mn(II) center. Taken together with our prior studies of the Mn(II)-chelating properties and antibacterial activity of a C-terminal tail mutant family, we propose that exclusion of water from the primary coordination sphere of the Mn(II) center is essential for Mn(II) sequestration. Lastly, pulse EPR spectroscopy enabled further characterization of how the CP-Ser –Ca coordinates Mn(II). Our spectroscopic results demonstrate that (i) CP forms a Mn(II)-His\textsubscript{6} coordination sphere at site 2 in the absence of Ca(II) and (ii) CP binds Mn(II) at additional sites in the absence of Ca(II). We propose that the canonical EF-hands coordinate Mn(II) under these conditions. CP (~Ca) therefore houses multiple low-affinity
Mn(II)-binding sites, and the addition of excess Ca(II) leads to the formation of a single high-affinity His6 coordination site.

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**Supporting Information.** Tables of protein nomenclature, mass spectrometry, crystal refinement statistics, bond lengths and angles. Figures of overlays of CP structures, additional CW EPR spectra, ENDOR and HYSCORE spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

**Conflict of Interest.** The authors declare no conflicts of interest.

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TOC Graphic

Mn(II)-His$_6$

Advanced EPR
Supporting Information for
Manganese Binding Properties of Human Calprotectin Under Conditions of High and Low Calcium: X-ray Crystallographic and Advanced EPR Spectroscopic Analysis

Derek M. Gagnon,¹ Megan Brunjes Brophy,² Sarah E. J. Bowman,²,³ Troy A. Stich,¹ Catherine L. Drennan,²,³,⁴ R. David Britt,¹,* and Elizabeth M. Nolan²,*

¹Department of Chemistry, University of California Davis, Davis, CA 95695, United States
²Department of Chemistry, ³Howard Hughes Medical Institute, and ⁴Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, United States

*Correspondence:
lnolan@mit.edu
Phone: 617-452-2495
Fax: 617-324-0505

rdbritt@ucdavis.edu
Phone: 530-752-6377
Fax: 530-752-8995
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Example MATLAB R2013a Code for Simulations Using EasySpin1

```matlab
matlabpool(4) % Allocate 4 processors for parallelization
Exp.mwFreq = 9.4; % GHz
Exp.Range = [100 600]; % mT
Exp.Temperature = 10; % K
Exp.nPoints = 2000;

% Generate vector of D values and initiate matrix of 0's to store calculated spectra
D = 285:5:685; % MHz
ys = zeros(length(D),exp.nPoints);

% Pre-generate all systems to be simulated, note only D is varied
for i = 1:length(D)
sys.S = 5/2;
sys.g = 2.001;
sys.lw = [.7 0]; % mT
sys.Nucs = '55Mn';
sys.A = 247; % MHz
sys.D = [D(i) .30*D(i)];
sys.DStrain = [10 10]; % MHz
sys.HStrain = 42; % MHz
syss(i) = sys;
clear sys
end

clear i

% Calculate spectra in parallel
parfor i = 1:length(D)
[-,ys(i,:)] = pepper(syss(i),exp);
end

clear exp i

% Scale by Gaussian Distribution and Sum
ysc = zeros(size(ys));
prob = zeros(1,length(D));
sigma = 400/(2*sqrt(2*log(2)));
N = 1/(sigma*sqrt(2*pi));
DO = (min(D)+max(D))/2;
for i = 1:length(D)
prob(i) = N*exp(-(D(i)-DO).^2/(2*sigma^2));
ysc(i, :) = prob(i)*ys(i, :);
end

clear i sigma N DO
ysim = zeros(1,length(ysc));
for i = 1:length(D)
ysim = ysim+ysc(i,:);
end

clear i

matlabpool close % Deallocates processors
```
### Table S1. Calprotectin protein nomenclature

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mutations</th>
<th>S100A8</th>
<th>S100A9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td>Wild-type</td>
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<td></td>
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<tr>
<td>CP-Ser</td>
<td>(C42S)</td>
<td></td>
<td>(C3S)</td>
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<tr>
<td>$^{15}$N-CP-Ser</td>
<td>(C42S) $^a$</td>
<td>(C42S)</td>
<td>(C3S)</td>
</tr>
<tr>
<td>CP-Ser ΔHis$_3$Asp</td>
<td>(C42S)(H83A)(H87A)</td>
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<tr>
<td>$^{15}$N-ΔHis$_3$Asp</td>
<td>(C42S)(H83A)(H87A)</td>
<td>(C3S)(H20A)(D30A)</td>
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<tr>
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<td>(C42S)(H83A)(H87A)</td>
<td>(C3S)(H20A)(D30A)</td>
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<tr>
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<td>(C3S)(H20A)(D30A)</td>
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<td>CP-Ser(H103A)</td>
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<td>(C42S)</td>
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<td>CP-Ser-AAA</td>
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<td>(C42S)</td>
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$^a$ Underlined subunits were expressed in M9 minimal medium containing $^{15}$NH$_4$Cl.

### Table S2. Mass spectrometric analysis of $^{15}$N-labeled CP and mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>S100A8 Calculated Mass (Da)</th>
<th>S100A8 Observed Mass (Da)</th>
<th>S100A9 Calculated Mass ± Met1 (Da)</th>
<th>S100A9 Observed Mass (Da)</th>
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</thead>
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<tr>
<td>$^{15}$N-CP-Ser</td>
<td>10 945.6</td>
<td>10 945.1</td>
<td>13 387.8</td>
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<tr>
<td>$^{15}$N-ΔHis$_3$Asp</td>
<td>10 809.5</td>
<td>10 808.9</td>
<td>13 255.7 (-Met1)</td>
<td>13 254.0</td>
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<tr>
<td>$^{15}$N(A8)-ΔHis$_3$Asp</td>
<td>10 809.5</td>
<td>10 808.6</td>
<td>13 143.6 (-Met1)</td>
<td>13 141.7</td>
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<tr>
<td>$^{15}$N(A9)-ΔHis$_3$Asp</td>
<td>10 686.3</td>
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<td>13 275.8</td>
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<td>12 984.7 (-Met1)</td>
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<td>13 143.6 (-Met1)</td>
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### Table S3. Crystallographic data and refinement statistics

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<th><strong>Mn(ll)-bound CP-Ser</strong></th>
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<tr>
<td><strong>Data collection</strong></td>
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<td>Wavelength (Å)</td>
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<td>Space group</td>
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<td><strong>Cell dimensions</strong></td>
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<td>$a$, $b$, $c$ (Å)</td>
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<tr>
<td>$\alpha$, $\beta$, $\gamma$ (°)</td>
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<td>Resolution (Å)</td>
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<td>No. unique reflections</td>
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<td>$CC_{1/2}$</td>
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<td>$R_{\text{sym}}$</td>
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<tr>
<td>$R_{\text{meas}}$</td>
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<tr>
<td>$\langle I \rangle / \sigma(\langle I \rangle)$</td>
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<td><strong>Refinement</strong></td>
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<tr>
<td>Resolution (Å)</td>
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<td>$R_{\text{cryst}}/R_{\text{free}}$</td>
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<td>$H_2O$</td>
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<td><strong>B-factors (Å²)</strong></td>
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<td>Chain A</td>
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<tr>
<td>Chain B</td>
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<tr>
<td>Chain C</td>
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<td>bond angles (°)</td>
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<td>allowed (%)</td>
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<td>favored (%)</td>
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*Highest resolution shell is shown in parentheses
**Bijvoet pairs were not merged and data were scaled anomalously
Table S4. Average Mn(II)–ligand distances at CP-Ser site 2

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Coordinating Atom</th>
<th>Mn(II)-L Distance (Å) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A8)His17</td>
<td>NE2</td>
<td>2.3 (0.05)</td>
</tr>
<tr>
<td>(A8)His27</td>
<td>NE2</td>
<td>2.3 (0.04)</td>
</tr>
<tr>
<td>(A9)His91</td>
<td>NE2</td>
<td>2.3 (0.04)</td>
</tr>
<tr>
<td>(A9)His95</td>
<td>NE2</td>
<td>2.3 (0.00)</td>
</tr>
<tr>
<td>(A9)His103</td>
<td>NE2</td>
<td>2.3 (0.04)</td>
</tr>
<tr>
<td>(A9)His105</td>
<td>NE2</td>
<td>2.3 (0.04)</td>
</tr>
</tbody>
</table>

a The distances (standard deviations) were determined using the metal ion geometry tool in Chimera.

Table S5. Average ligand(NE2)–Mn–ligand(NE2) angles at CP-Ser site 2 a

<table>
<thead>
<tr>
<th></th>
<th>(A8)His17</th>
<th>(A8)His27</th>
<th>(A9)His91</th>
<th>(A9)His95</th>
<th>(A9)His103</th>
<th>(A9)His105</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A8)His17</td>
<td>n. a.</td>
<td>93.5 (1.3)</td>
<td>88.6 (0.9)</td>
<td>175.2 (1.5)</td>
<td>87.2 (1.4)</td>
<td>88.5 (2.5)</td>
</tr>
<tr>
<td>(A8)His27</td>
<td>-</td>
<td>n. a.</td>
<td>89.1 (2.3)</td>
<td>89.3 (1.7)</td>
<td>94.7 (3.0)</td>
<td>176.3 (1.8)</td>
</tr>
<tr>
<td>(A9)His91</td>
<td>-</td>
<td>-</td>
<td>n. a.</td>
<td>87.8 (1.4)</td>
<td>174.3 (2.6)</td>
<td>93.0 (1.3)</td>
</tr>
<tr>
<td>(A9)His95</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>n. a.</td>
<td>96.0 (2.2)</td>
<td>88.0 (3.2)</td>
</tr>
<tr>
<td>(A9)His103</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>n. a.</td>
<td>83.3 (1.7)</td>
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<tr>
<td>(A9)His105</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>n. a.</td>
</tr>
</tbody>
</table>

a The bond angles (standard deviations) were determined using the metal ion geometry tool in Chimera.
<table>
<thead>
<tr>
<th>EF-hand</th>
<th>Amino Acid</th>
<th>Coordinating Group</th>
<th>Ca(II)–L or Na(I)–L Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8, N-terminal</td>
<td>Ser20</td>
<td>C=O</td>
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<td>C=O</td>
<td>2.3</td>
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<td></td>
<td>Asn25</td>
<td>C=O</td>
<td>2.4</td>
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<tr>
<td></td>
<td>Ala28</td>
<td>C=O</td>
<td>2.3</td>
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<td>Water</td>
<td>O</td>
<td>2.6</td>
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<td>Oε2</td>
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<td>A9, N-terminal</td>
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<td>Oε2</td>
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Figure S1. SDS-PAGE (15% Tris-HCl) of CP-Ser and mutants expressed in M9 minimal medium. (A) New England Biolabs P7702S protein marker (lane A), $^{15}$N-CP-Ser (lane B), $^{15}$N(A8)-ΔHis$_3$Asp (lane C). $^{15}$N(A9)-ΔHis$_3$Asp (lane D). (B) New England Biolabs P7702S protein marker (lane A), $^{15}$N-ΔHis$_3$Asp (lane B).
Figure S2. Overlay of the crystal structure of Ca(II)- and Mn(II)-bound CP-Ser (green/blue ribbons with side chains in orange) with PDB 1XK4\textsuperscript{S1} (grey). (A) Models of the $\alpha\beta$ dimers. The Ca(II) ions (yellow spheres), Na(I) ions (purple spheres), and Mn(III) ions (magenta spheres) are taken from the Ca(II)- and Mn(II)-bound structure of CP-Ser determined in this work. (B) The His$_3$Asp motifs. (C) The His$_4$ motif and the Mn(II)-His$_6$ site.
Figure S3. Overlay of the crystal structure of Ca(II)- and Mn(II)-bound CP-Ser (green/blue ribbons with side chains in orange) with PDB 4GGF52 (grey). (A) Models of the αβ dimers. The Ca(II) ions (yellow spheres), Na(I) ions (purple spheres), and Mn(II) ions (magenta spheres) are taken from the Ca(II)- and Mn(II)-bound structure of CP-Ser presented in this work. (B) The His$_3$Asp sites. The Mn(II) ions from the 4GGF structure are omitted for clarity. (C) The Mn(II)-His$_6$ sites.
**Figure S4.** Electron density of Mn(II), Ca(II), and Na(I) ions. (A) and (B) Composite omit map (2σ) of ions coordinated to the His$_6$ site and the A8 EF-hands or the His$_3$Asp site and A9 EF-hands. Non-canonical N-terminal E-F hand sites are shown with Na(I) and the canonical C-terminal EF-hand sites with Ca(II). (C) and (D) Anomalous electron density (λ = 1.54 Å) contoured at 4σ. (E) and (F) Anomalous electron density (λ = 1.54 Å) contoured at 2σ. A8 and A9 subunits are shown in green and blue, respectively. Residues are shown as orange sticks, with Mn(II) ions as magenta spheres, Ca(II) ions as yellow spheres, and Na(I) ions as purple spheres. Composite omit maps are shown in wheat mesh, and anomalous electron density is shown as pink mesh.
Figure S5. CW EPR spectra of Mn(II)- and Ca(II)-bound CP-Ser and C-terminal tail mutants H103A, H104A, H105A, AHA, and AAA. Spectrometer settings: temperature, 10 K; microwaves, 0.2 mW at 9.4 GHz; modulation, 0.5 mT at 100 kHz. Sample conditions: 200 μM CP, 2 mM Ca(II), and 180 μM Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).
Figure S6. Q-band Mims-ENDOR of Mn(II)- and Ca(II)-bound CP-Ser collected with different tau values. The black lines are simulations using $S' = 1/2$ (dashed) or $S = 5/2$ (solid). Spectrometer settings: temperature, 10 K; microwaves, 34.1 GHz; $B_0 = 1222.5$ mT, 8 ns $\pi/2$. Sample conditions: 200 $\mu$M CP, 2 mM Ca(II), and 180 $\mu$M Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).
Figure S7. Q-band HYSCORE of Ca(II)- and Mn(II)-bound ΔHis₃Asp at natural abundance.

Spectrometer settings: temperature, 10 K; microwaves, 34.1 GHz; τ = 200 ns; T₀ = 20 ns; π = 16 ns; π/2 = 8 ns. Sample conditions: 200 μM CP, 2 mM Ca(II), and 180 μM Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).
Figure S8. Q-band HYSCORE of Ca(II)- and Mn(II)-bound $^{15}$N-$\Delta$His$_3$Asp with $^{15}$N-enriched S100A8 and S100A9. Spectrometer settings: temperature, 10 K; microwaves, 34.1 GHz; $\tau = 200$ ns; $T_0 = 20$ ns; $\pi = 16$ ns; $\pi/2 = 8$ ns. Sample conditions: 200 $\mu$M CP, 2 mM Ca(II), and 180 $\mu$M Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).
Figure S9. Q-band HYSCORE of Ca(II)- and Mn(II)-bound $^{15}$N(A8)-ΔHis$_3$Asp with $^{15}$N-enriched S100A8. Spectrometer settings: temperature, 10 K; microwaves, 34.1 GHz; $\tau = 200$ ns; $T_0 = 20$ ns; $\pi = 16$ ns; $\pi/2 = 8$ ns. Sample conditions: 200 μM CP, 2 mM Ca(II), and 180 μM Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).
Figure S10. Q-band HYSCORE of Ca(II)- and Mn(II)-bound $^{15}$N(A9)-ΔHis$_3$Asp with $^{15}$N-enriched S100A9. Spectrometer settings: temperature, 10 K; microwaves, 34.1 GHz; $\tau = 200$ ns; $T_0 = 20$ ns; $\pi = 16$ ns; $\pi/2 = 8$ ns. Sample conditions: 200 μM CP, 2 mM Ca(II), and 180 μM Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).
Figure S11. Q-band proton Davies ENDOR of Ca(II)- and Mn(II)-bound CP-Ser. Spectrometer settings: temperature, 4.5 K; microwaves, power at 34.1 GHz; $\tau_{\text{inv}} = 64$ ns, $\pi/2 = 8$ ns, $\pi = 16$ ns, $\tau_{\text{RF}} = 20$ $\mu$s. Sample conditions: 200 $\mu$M CP-Ser, 2 mM Ca(II), 180 $\mu$M Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).
Figure S12. Room-temperature EPR provides detection of free Mn(II) in samples of Mn(II)-bound CP-Ser prepared in the absence of Ca(II). The data was smoothed with a Savitzky-Golay filter (10 pt window, 6th order polynomial) and linear baseline corrected. Double integration was performed on this data and compared to a doubly integrated spectrum of a 200 μM Mn(II) standard (data not shown) to quantify the concentration of spins. For reference a 20 μM Mn(II) in buffer solution is shown in red. Spectrometer settings: νmw = 9.8 GHz, modulation = 0.5 mT at 100 kHz, power = 0.2 mW. Sample conditions for the 0.18:1.0 sample: 1.0 mM CP-Ser, 180 μM Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200). Sample conditions for the 0.9:1.0 sample: 200 μM CP-Ser, 180 μM Mn(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200).
Figure S13. Relative $^{14}$N modulation depths in 3-pulse ESEEM for CP-Ser in the absence and presence of Ca(II). The blue and red traces are the $^{14}$N modulation depths for 0.9:1.0 and 0.18:1.0 Mn(II):CP ratios in the absence of Ca(II), respectively. The green trace is the $^{14}$N modulation depth in the presence of 10 equivalents of Ca(II). The grey trace is a 0.50 scaling of the green +Ca(II) trace. Spectrometer settings: $\nu_{mw} = 9.525(5)$ GHz, $B_0 = 327.35$ mT, 8 ns $\pi/2$, $\tau = 216$ ns, $T_0 = 80$ ns, temperature = 10 K. Sample conditions (-Ca): 180 $\mu$M Mn(II) with either 200 $\mu$M or 1.0 mM CP-Ser at pH 7.5 (75 mM HEPES, 100 mM NaCl, 20% (v/v) PEG 200). The +Ca samples were prepared in the same buffer and contained 180 $\mu$M Mn(II), 200 $\mu$M CP-Ser, and 1.0 mM Ca(II)
**Figure S14.** Representative Mn(II) simulations with various axial zero-field splitting values ($D$). All spectra were simulated using $g = 2.001$, natural linewidth of 0.5 mT, $S = 5/2$, 40 MHz of $H$ strain, and 33% $D$ and $E$ strain. The black traces have a $D$ value of 500 MHz with an $E/D$ of 0.30 serving as an approximation for Mn(II) bound to the His$_6$ site of CP. The blue traces have varying $D$ values labeled near each trace with an $E/D$ ratio of 0.20. Each pair of spectra have a 1:1 weighting of the low $D$ (500 MHz) to high $D$ ($\geq$ 1000 MHz).
Supporting References


Biographical Note

Megan Brunjes Brophy was born in Portland, Oregon to Crea Williams and David Baker. She was raised in Newport, Oregon where she attended Newport High School and spent her weekends volunteering at the Oregon Coast Aquarium and the Hatfield Marine Science Center. In 2006, Megan matriculated to Reed College and graduated with a B. A. in Chemistry in 2010. While at Reed, she performed research with Professors Arthur Glasfeld and Margret Geselbracht, and wrote her undergraduate thesis on the molecular basis for metal-ion selectivity of the manganese transport regulator (MntR) from *Bacillus subtilis*. Megan came to MIT in the fall of 2010, where she studied metal-ion sequestration by the human host-defense protein calprotectin with Professor Elizabeth Nolan. Following graduation, the author plans to pursue postdoctoral studies in the laboratory of Professor Michael Marletta at UC Berkeley. Megan enjoys coffee, feminism, and long walks on the beach.
EDUCATION

Massachusetts Institute of Technology Cambridge, Massachusetts
Ph. D., Biological Chemistry, June 2015
Dissertation Title: Bioinorganic Chemistry of the Human Host-Defense Protein Calprotectin
Advisor: Professor Elizabeth M. Nolan

Reed College Portland, Oregon
B.A., Chemistry, May 2010
Senior Thesis Title: Going Through the Motions: Structure and Dynamics of MntR
Advisor: Professor Arthur Glasfeld

HONORS AND FELLOWSHIPS

09/14 NIH Travel Award to the International Society of Zinc Biology meeting
08/13 Department of Chemistry Award for Outstanding Teaching (MIT)
04/13 Morse Travel Grant, for attendance to the 2013 Cell Biology of Metals Gordon Research Conference (MIT, Chemistry Department)
06/12-08/12 Stephen J. Lippard Summer Graduate Fellowship (MIT)
03/12 National Science Foundation Graduate Research Fellowship Program Honorable Mention
09/10-06/11 Robert T. Haslam Presidential Fellowship (MIT)
09/10-08/11 Chemistry Biology Interface Training Program Fellowship (MIT)
08/09-05/10 F.W. Erickson Scholarship (Reed College, granted to female student majoring in chemistry selected for academic excellence)
08/08-05/09 Award for Academic Excellence awarded by the Division of Mathematics and Natural Sciences of Reed College

PUBLICATIONS


PRESENTATIONS
Deciphering the Zinc Sequestration Abilities of Host-Defense Proteins of the S100 Family. Presented at the 2014 meeting of the International Society for Zinc Biology (invited talk); Forest Gove, CA; September 16, 2014.

Elucidating the Molecular Basis for Mn(II) and Zn(II) Sequestration by the Human Innate Immune System. Presented in the Massachusetts Institute of Technology Chemistry Student Seminar Series (talk); Cambridge, MA; May 17, 2013.


Co(II) as a Spectroscopic Probe to Study the Metal Binding Properties of Calprotectin. Presented in the Smith College Department of Chemistry Lunchbag Series (invited talk); Northampton, MA; October 17, 2012.

Utilizing Fluorescent Sensors to Investigate the Metal Binding Properties of Human Calprotectin. Presented at Strem Chemicals (invited talk); Newburyport, MA; August 15, 2012.


