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Manganese and Microbial Pathogenesis: Sequestration by the Mammalian Immune System and Utilization by Microorganisms

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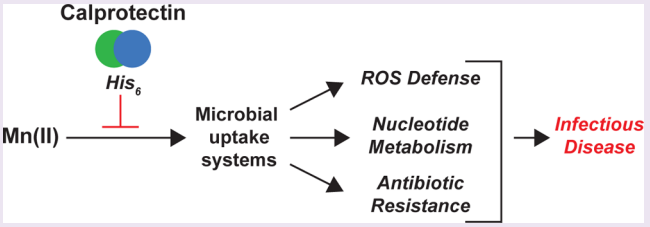
ABSTRACT: Bacterial and fungal pathogens cause a variety of infectious diseases and constitute a significant threat to public health. The human innate immune system represents the first line of defense against pathogenic microbes and employs a range of chemical artillery to combat these invaders. One important mechanism of innate immunity is the sequestration of metal ions that are essential nutrients. Manganese is one nutrient that is required for many pathogens to establish an infective lifestyle. This review summarizes recent advances in the role of manganese in the host–pathogen interaction and highlights Mn(II) sequestration by neutrophil calprotectin as well as how bacterial acquisition and utilization of manganese enables pathogenesis.



Metals ions are essential for the proliferation of all kingdoms of life and perform a variety of structural and chemical tasks. In order to establish a virulent lifestyle, pathogens must acquire and concentrate metal ions from the host.[1−3] The mammalian innate immune system has evolved to sequester bioavailable iron, manganese, and zinc at sites of infection and thereby prevent microbial growth.[4,5] The competition between host and pathogen for these essential nutrients is an important facet of health and disease progression. Bioinorganic chemistry underlies this aspect of innate immunity and microbial pathogenesis. Elucidating the coordination chemistry of both mammalian and microbial proteins involved in the sequestration, recruitment, and utilization of these metals provides a molecular foundation for physiology and informs therapeutic development. This review highlights recent chemical and biological insights into the battle between host and microbe for manganese. The manganese-sequestering host-defense protein calprotectin (CP) as well as the bacterial Mn(II) uptake systems and processes that require Mn(II) and contribute to pathogenesis are discussed.

A historical paradigm for metal-ion withholding by the host immune system is provided by studies of iron-binding proteins. The host proteins transferrin, lactoferrin, and siderocalin (also named lipocalin-2 or neutrophil gelatinase-associated lipocalin (NGAL)) deplete bioavailable iron in blood plasma, secretary fluids, and at sites of infection and thereby limit the availability of iron to microorganisms (Figure 1).[6−8] Transferrin and lactoferrin coordinate Fe(III) with high affinity, whereas siderocalin captures iron-bound enterobactin. These systems are detailed in several prior reviews[3,9,10] and provide inspiration for investigations of other metal-chelating host-defense proteins.

Although less celebrated than iron for many years, manganese and zinc are important nutrients at the host–pathogen interface.[5] In this context, members of the S100 family of Ca(II)-binding proteins have emerged as contributors to transition-metal-ion homeostasis and players in innate immunity. Select family members coordinate Mn(II), Cu(II), and Zn(II) and contribute to the host metal-withholding response (Figure 1).[11−14] The human proteins S100A7 (23 kDa) and S100A12 (21 kDa) are homodimers that each house two interfacial His_3Asp metal-ion binding motifs.[13,15,16] Human S100A7, also called psoriasin, is expressed by epithelial tissues and displays Zn(II)-reversible growth inhibition of *Escherichia coli*.[12,13,17,18] Human S100A12 is a Zn(II)- and Cu(II)-binding neutrophil protein that exhibits chemotactic properties.[15,16,19] Despite structural characterization,[13,16] the bioinorganic chemistry of S100A7 and S100A12 remains poorly understood, and elucidating how these host-defense proteins contribute to metal homeostasis in broad terms is an important avenue for future work.

CP, a heterooligomer of S100A8 (10.8 kDa, also named MRP8 or calgranulin A) and S100A9 (13.2 kDa, also named MRP14 or calgranulin B),[20] is another S100 family member that contributes to innate immunity. In early studies, CP was identified as a Zn(II)-reversible antibacterial agent in human neutrophil lysates.[21,22] Subsequently, a role for CP in Mn(II) homeostasis was discovered.[11] To date, CP is the only known mammalian Mn(II)-sequestering host-defense protein, and additional biomolecules that modulate Mn(II) availability at sites of infection may be uncovered in further discovery efforts. CP is reported to constitute ca. 40% of the total cytoplasmic protein in neutrophils,[23] and CP concentrations exceeding 1 mg mL^{-1} (ca. 50 μM) have been found in infected tissues.[24] CP is therefore an abundant and important component of the innate immune response and a significant focus of recent work

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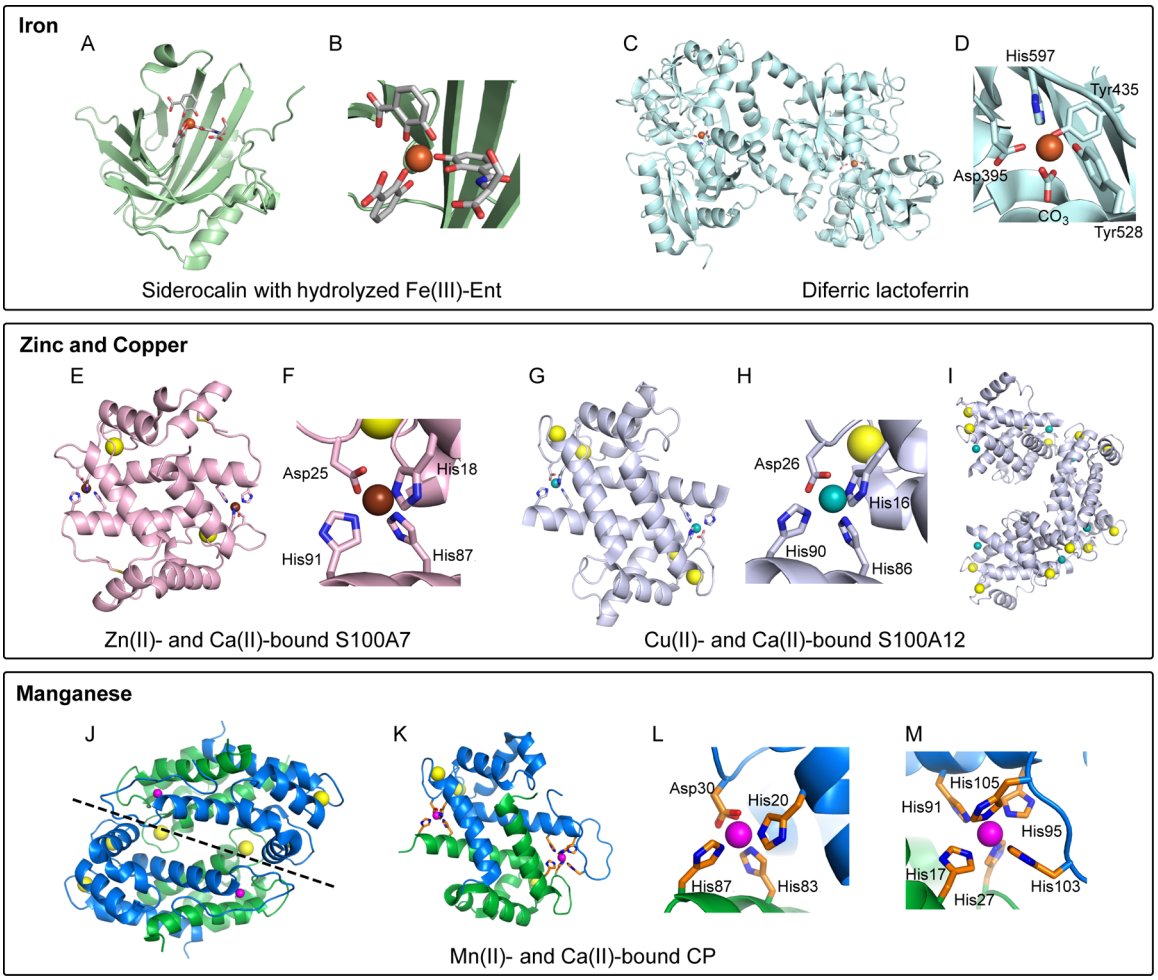

Figure 1. Crystal structures of metal-binding human host-defense proteins. (A, B) Siderocalin with hydrolyzed ferric enterobactin bound (PDB: 1L6M).[7] (C, D) The diferric form of lactoferrin with the Fe(III)-binding site of the C-terminal lobe shown (PDB: 1LFG).[25] Iron(III) ions are shown as orange spheres. (E, F) The Zn(II)- and Ca(II)-bound form of human S100A7 (PDB: 2PSR).[13] The Zn(II) ions are shown as chocolate spheres. (G–I) The Cu(II)- and Ca(II)-bound form of human S100A12 (PDB: 1ODB).[16] Panel I shows the packing of three S100A12 homodimers in the asymmetric unit. Copper ions are shown as teal spheres. (J–M) The Mn(II)- and Ca(II)-bound form of human CP (PDB: 4GGF).[26] S100A9 subunits are colored blue, and S100A8 subunits are colored green. The $\alpha_2\beta_2$ tetramer (J) and an $\alpha\beta$ heterodimer unit (K) taken from the $\alpha_2\beta_2$ tetramer are shown. Mn(II), shown as pink spheres, is coordinated by site 1 (L, 50% occupancy) and site 2 (M, 100% occupancy). There are three Ca(II) ions per $\alpha\beta$ dimer. For S100A7, S100A12, and CP, the Ca(II) ions are represented as yellow spheres. No structure of the Zn(II)-bound form of CP has been reported.

on Mn(II) homeostasis at the host–pathogen interface as described below.

COORDINATION CHEMISTRY OF HUMAN CALPROTECTIN

Human CP exhibits remarkable biological coordination chemistry that results from its heterooligomeric structure. It is a heterodimer or -tetramer of S100A8 (α) and S100A9 (β) and exists as either an $\alpha\beta$ heterodimer or an $\alpha_2\beta_2$ heterotetramer.[20,27,28] Each subunit contains two Ca(II)-binding EF-hand domains, and Ca(II) binding modulates the quaternary structure of CP by mediating the conversion of the dimer to the tetramer form.[27] CP displays two transition metal-ion binding motifs per S100A8/S100A9 heterodimer, both of which are located at the heterodimer interface (Figure 1).[20] Site 1 is a His_3Asp motif that is formed by residues His83 and His87 from S100A8 and residues His20 and Asp30 from S100A9. Site 2 is an unusual His_4 or His_6 motif formed by residues His17 and His27 from S100A8 and His91 and His95 from S100A9. The His_6 motif is completed by His103 and His105 from the S100A9 C-terminal tail.[26,29] The Zn(II)- and Mn(II)-binding properties of CP have been studied extensively and are the focus of this review. Both sites 1 and 2 coordinate Zn(II) with high affinity, whereas only the His_6 motif of site 2 provides high-affinity Mn(II) complexation.[26,29−32] The metal-ion affinities of both sites are Ca(II)-dependent, and CP morphs into a high-affinity chelator when in the presence of excess Ca(II).[31,32]

Considerations for Metal-Binding Studies. CP provides a unique coordination chemistry puzzle, and challenges associated with investigating its Zn/Mn-binding properties include: (i) each CP $\alpha\beta$ unit has six unique metal-binding sites, (ii) Ca(II) ions cause changes in quaternary structure and transition-metal affinities, and (iii) CP binds Zn(II) and Mn(II) with high affinity. In terms of the third point, obtaining quantitative information about the metal-binding affinities (as measured by apparent dissociation constant, herein K_d) is critical for understanding how CP sequesters metal ions in biological contexts. Several methods, including isothermal

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Table 1. Reported Apparent Dissociation Constant Values (K_d) for Human CP and Experimental Methods[a]

metal	protein	dissociation constant (K_d)	method	buffer conditions	ref
Zn(II)	CP-Ser[b]	$K_{d,\ site1}$ = 133 ± 58 pM $K_{d,\ site2}$ = 185 ± 219 nM	Competition[c]	75 mM HEPES, 100 mM NaCl, pH 7.5	31
Zn(II)	CP-Ser[b]	$K_{d,\ site1} \leq$ 10 pM $K_{d,\ site2} \leq$ 240 pM	Competition[c]	75 mM HEPES, 100 mM NaCl, pH 7.5 20 equivalents Ca(II) / CP ($\alpha\beta$)	31
Zn(II)	CP	K_{d1} = 1.4 nM[d] K_{d2} = 5.6 nM[d]	ITC[e]	20 mM Tris, 100 mM NaCl, pH 7.5 stoichiometric Ca(II)[f] 5 mM β-mercaptoethanol	30
Zn(II)	ΔHis_3Asp[b]	$K_{d,\ site2}$ = 3.4 ± 1.2 nM	ITC[g]	20 mM HEPES, 75 mM NaCl, pH 7.5 stoichiometric Ca(II)[f]	26
Zn(II)	ΔHis_4[b]	$K_{d,\ site1}$ = 8.2 ± 1.5 nM	ITC[g]	20 mM HEPES, 75 mM NaCl, pH 7.5 stoichiometric Ca(II)[f]	26
Mn(II)	CP-Ser[b]	$K_{d,\ site2}$ = 4.9 ± 1.0 μM K_d = 1.0 mM (n=2)	EPR[h]	75 mM HEPES, 100 mM NaCl, pH 7.5	32
Mn(II)	CP-Ser[b]	$K_{d,\ site2}$ = 194 ± 203 nM $K_{d,\ site1}$ = 21 ± 5 μM	EPR[h]	75 mM HEPES, 100 mM NaCl, pH 7.5 40 equivalents Ca(II) / CP ($\alpha\beta$)	32
Mn(II)	CP-Ser[b]	$K_{d,\ site2}$ > 550 nM	Competition[i]	75 mM HEPES, 100 mM NaCl, pH 7.5	32
Mn(II)	CP-Ser[b]	$K_{d,\ site2}$ < 550 nM	Competition[i]	75 mM HEPES, 100 mM NaCl, pH 7.5 40 equivalents Ca(II) / CP ($\alpha\beta$)	32
Mn(II)	CP	K_{d1} = 1.3 nM[d] K_{d2} = 3.7 μM[d]	ITC[e]	20 mM Tris, 100 mM NaCl, pH 7.5 stoichiometric Ca(II)[f] 5 mM β-mercaptoethanol	30
Mn(II)	ΔHis_3Asp[b]	$K_{d,\ site\ 2}$ = 5.8 ± 1.6 nM	ITC[g]	20 mM HEPES, 75 mM NaCl, pH 7.5 stoichiometric Ca(II)[f]	26

[a]See section entitled "Considerations for Metal-Binding Studies" for further discussion. [b]CP contains two native cysteine residues that were mutated to serine for these metal-binding studies. [c]Competition titrations were performed with ZP4 at 25 °C. [d]The K_d values were not assigned to the metal-binding sites. [e]Direct ITC titrations were performed at 30 °C. Stoichiometric Mn(II)/Zn(II) binding was observed. [f]The definition of stoichiometric Ca(II) in terms of molar equivalents per CP unit or Ca(II)-binding site is unclear. [g]Direct ITC titrations were performed at 25 °C. Stoichiometric Zn(II)/Mn(II) binding was observed. [h]Direct EPR titrations were performed at room temperature. The +Ca(II) titrations are limited by the concentrations required for detectable Mn(II). [i]Competition titrations were performed with ZP1 at 25 °C.

titration calorimetry (ITC),[2] electron paramagnetic resonance spectroscopy (EPR),[33] and competition titrations,[34] have been employed to probe the Zn/Mn affinities of CP (Table 1). For a high-affinity chelator like CP, stoichiometric binding occurs and direct titrations performed by any method can afford only an upper limit ($K_d \sim 10^{-8}$ M) to the K_d value.[2,34] Competition titrations are one approach to overcoming this problem, making small-molecule metal sensors with known K_d values valuable. However, the competing sensor must have an appropriate K_d value to probe CP and not succumb to artifacts in the presence of protein.[34]

CP Binds Zinc with High Affinity. CP binds two equivalents of Zn(II) with high affinity (Table 1).[30,31] The Ca(II)-insensitive Zn(II) sensor ZP4 (K_d = 0.65 nM)[35] revealed that CP utilizes Ca(II) ions to modulate its affinity for Zn(II) at both sites 1 and 2.[31] On the basis of ZP4 competitions, in the absence of Ca(II), CP ($\alpha\beta$) binds Zn(II) with $K_{d,site1}$ = 133 ± 58 pM and $K_{d,site2}$ = 185 ± 219 nM. In the presence of excess Ca(II), CP ($\alpha_2\beta_2$) effectively outcompetes ZP4 for two equivalents of Zn(II), preventing the determination of reliable dissociation constants. Nevertheless, upper limits were ascertained to be $K_{d,site1} \leq$ 10 pM and $K_{d,site2} \leq$ 240 pM. Further investigations are required to obtain more accurate Zn(II) K_d values and to elucidate the Zn(II) coordination numbers and geometries at both sites.

CP Binds Manganese(II) with High Affinity. CP is unique among S100 proteins, and known host-defense proteins, because of its ability to sequester Mn(II). CP therefore overcomes the kinetic lability of this $3d^5$ metal ion.[29] The Mn(II) affinity of CP has been investigated by using ITC, room-temperature EPR (RT-EPR), and competition titrations.[26,29,30,32] Taken together, the results from these experiments demonstrate that CP has the capacity to coordinate Mn(II) with remarkably high affinity for a Mn-binding protein (Table 1).[32] A combination of RT-EPR and Mn(II) competition titrations using the Ca(II)-insensitive metal sensor ZP1 ($K_{d1,Mn}$ = 550 nM)[36] revealed that CP uses Ca(II) ions to modulate its Mn(II)-binding properties.[32] For example, in the absence of Ca(II), CP ($\alpha\beta$) exhibits relatively low affinity for Mn(II) and does not compete with ZP1 for Mn(II). The presence of excess Ca(II) ions enhances the Mn(II) affinity of CP, and enables CP ($\alpha_2\beta_2$) to sequester Mn(II) from ZP1. Thus, Ca(II) binding enhances the Mn(II) affinity and appears to be a general strategy for tuning the metal-sequestering capability and antibacterial activity of CP (*vide infra*). On the basis of current data (Table 1), the upper limit to the K_d value of CP for Mn(II) in the presence of Ca(II) is in the nanomolar range. Given the Ca(II)-dependent Mn(II) affinities, we reason that Ca(II)-bound CP is required for Mn(II) scavenging in biological contexts. Site 2 is the high-affinity Mn(II) site,[32] and both crystallographic and solution studies established that CP employs a hexahistidine motif for Mn(II) complexation at site 2 (Figures 1M, 2A).[26,29] Site 1, which exhibits the same primary coordination sphere as Mn-containing superoxide dismutases (SODs) (Figures 1L, 2G), has relatively low affinity for Mn(II). Whether site 1 chelates Mn(II) in physiological contexts is unclear. Its Mn(II) affinity is too low for Mn(II) sequestration;

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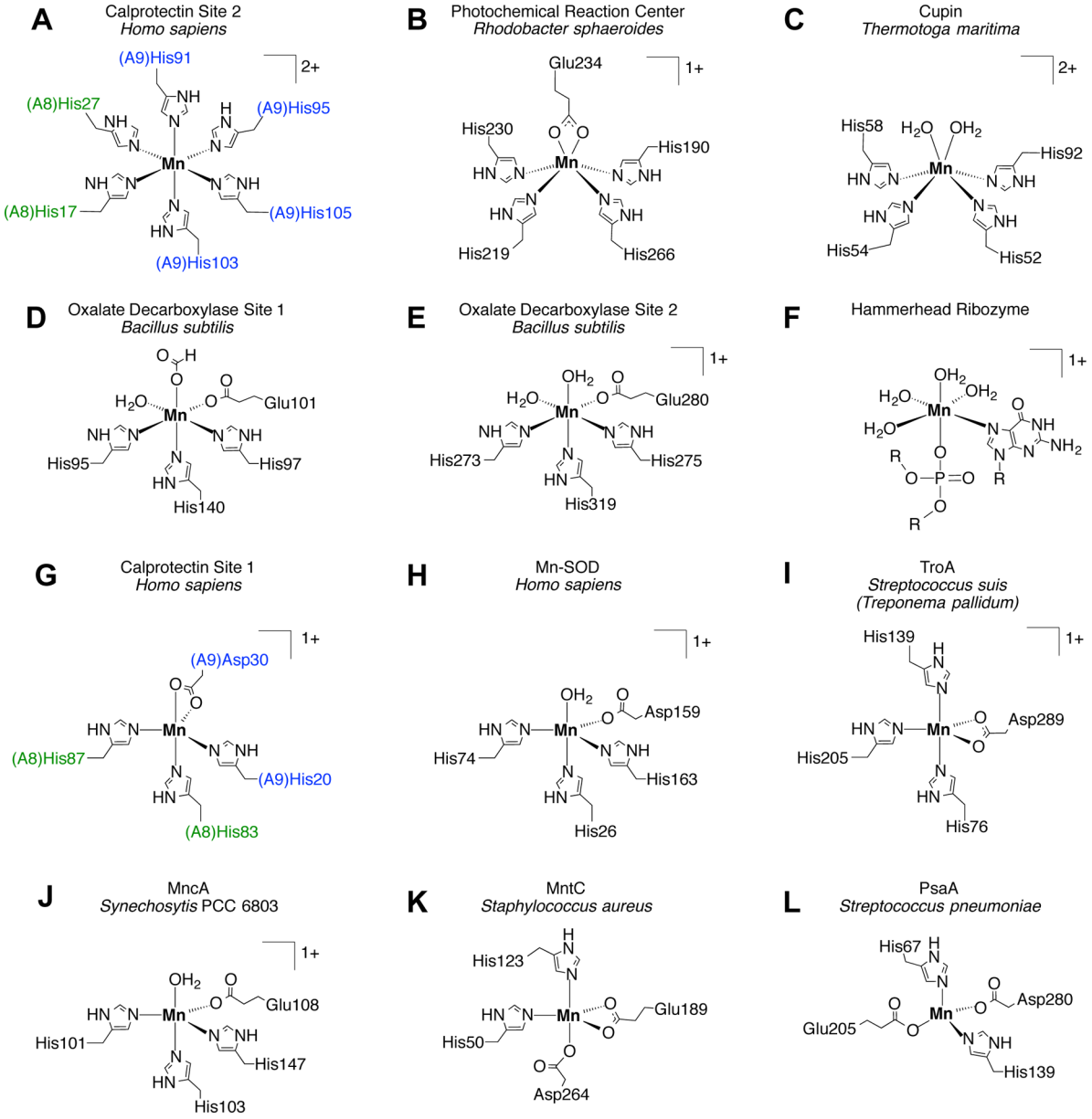

Figure 2. Examples of mononuclear biological Mn(II) sites. (A) The His_6 site of human calprotectin.[26,29] (B) The photochemical reaction center of *Rhodobacter sphaeroides*.[37] (C) A cupin of unknown function from *Thermotoga maritima*.[38] (D) Site 1 of the *Bacillus subtilis* oxalate decarboxylase.[39] (E) Site 2 of the *B. subtilis* oxalate decarboxylase.[39] (F) The hammerhead ribozyme.[40] (G) The His_3Asp site of human calprotectin.[26,32] (H) The primary coordination sphere of Mn-SOD.[41] (I) The TroA solute binding protein from *Streptococcus suis* and *Treponema pallidum*.[42,43,59] The numbering for the *S. suis* TroA is shown. Both TroA proteins have been crystallized in the Zn(II)-bound forms, but biochemical data indicate that these proteins transport Mn(II). (J) MncA from *Synechosytis* PCC 6803.[44] (K) The Mn(II)-solute binding protein of *Staphylococcus aureus*.[45] (L) The pneumococcal surface antigen (PsaA) from *Streptococcus pneumoniae* depicted with a four-coordinate geometry.[46]

however, it is possible that site 1 contributes to Mn(II) buffering.

The Hexahistidine Motif and the S100A9 C-terminal Tail. From the standpoint of biological Mn(II) coordination chemistry, CP is a notable example for several reasons. Mn(II) coordination at interfacial protein sites is unusual.[47] The hexahistidine site (Figure 1M) is unprecedented among known metalloproteins and provides Ca(II)-dependent Mn(II) sequestration. Most biological Mn(II) coordination spheres provide a mixture of nitrogen- and oxygen-based ligands (Figure 2), as expected on the basis of hard-soft acid-base theory. Thus, it is surprising that CP exclusively utilizes neutral His ligands to coordinate Mn(II). Formation of the His_6 site results in organization of the C-terminal tail of S100A9,[26] and the residues His103 and His105 complete the octahedral coordination sphere.[26,29] Mutant proteins that lack histidine moieties at positions 103 and/or 105 of the S100A9 C-terminal tail bind Mn(II) at site 2, but these mutants appear to be unable to sequester this metal.[26,29] Additional structural and spectroscopic investigations are required to elucidate how the CP scaffold and His_6 site are tuned to capture Mn(II).

Metal Binding Accounts for CP Antimicrobial Activity. CP has broad-spectrum *in vitro* antimicrobial activity attributed to its ability to starve microbes of essential nutrient metals following its release into the extracellular space.[11,24,26,31,48] *In vitro* studies of CP revealed that (i) the metal-binding sites are

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important for antibacterial activity[30,31] and (ii) this activity is enhanced in the presence of 2 mM Ca(II).[31] Taken together with the metal-binding studies described above, these results provide a working model whereby CP employs physiological Ca(II) ion gradients to modulate its metal affinities and antibacterial activity.[31] When stored in the cytosol of neutrophils or epithelial cells, CP encounters relatively low Ca(II) concentrations (nanomolar) under resting conditions. Following release into the extracellular milieu, CP encounters Ca(II) concentrations in the low-millimolar range and morphs into a potent chelator. CP has been detected at sites of infection in concentrations as high as ca. 50 μM,[24] suggesting that sufficient CP is available to chelate bioavailable metals in these environments.

Studies of CP mutant proteins and numerous bacterial strains revealed that site 2 contributes to *in vitro* growth inhibitory activity more than site 1.[26,29,31] The precise origins for this behavior are unclear and require further investigation.[29] Although site 2 coordinates Mn(II) with high affinity, it has a thermodynamic preference for Zn(II), and other as-yet unappreciated factors may be at work.[29,31,32]

BACTERIAL MANGANESE(II) ACQUISITION AND COMPETITION WITH CP

With the discoveries that CP sequesters and depletes Mn(II) at sites of infection,[11] the Mn(II) biology of pathogens and commensal microbes is a critical area of investigation.[2] The mechanisms of Mn(II) acquisition by microbes as well as the microbial processes and virulence factors that require Mn(II) are of particular interest (Figure 3).[2] Indeed, animal models of infection suggest that Mn(II) uptake systems are required for virulence in a variety of bacterial pathogens including *Staphylococcus aureus*,[49] *Streptococcus pneumoniae*,[50,51] *Borrelia burgdorferi*,[52] *Salmonella* Typhimurium,[53] and *Yersinia pestis*.[54]

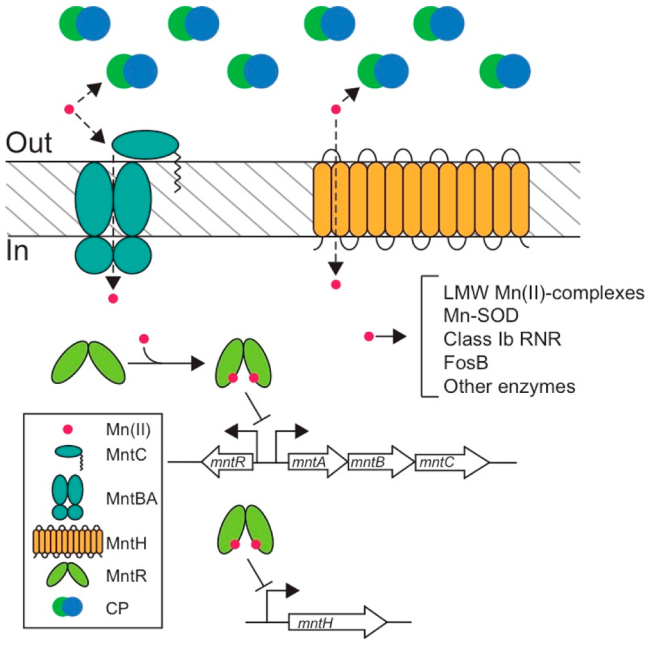

Figure 3. Model of Mn(II) homeostasis in *S. aureus* and Mn(II)-dependent processes that CP may disrupt. CP released into the extracellular space coordinates Mn(II) at sites of infection and competes with the staphylococcal Mn(II) transporters MntC and MntH. Following uptake, Mn(II) is incorporated into metalloenzymes including MnSOD, Class Ib RNR, and FosB. Expression of MntABC and MntH is downregulated under Mn(II)-rich conditions by the metalloregulatory protein MntR.

Bacteria primarily rely on two classes of Mn(II) importers, Nramp-type transporters and ATP-binding casette (ABC) importers, to shuttle divalent manganese into the cytoplasm.[55] The Nramp-type transporters (e.g., MntH from *S. aureus*, Figure 3) are composed of multiple membrane-embedded helices. A paucity of structural or biochemical information about these transporters is available,[56] making studies of Nramp-type machinery a rich area for exploration. 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 *S. pneumoniae* and MntABC of *S. aureus*. In recent years, structural and biochemical studies of the solute-binding proteins from *S. pneumoniae* (PsaA) and *S. aureus* (MntC) have informed the mechanism of bacterial Mn(II) capture.[45,46,57,58] Structural studies revealed that various Mn(II) solute-binding proteins display nearly identical secondary and tertiary structures.[42,45,46,59,60] These solute-binding proteins are characterized by two $(\alpha/\beta)_4$ lobes that are linked by a backbone α-helix (Figure 4). 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. No Mn(II) solute-binding protein has been crystallized with its transmembrane partner, and the mechanism of metal ion release into the transmembrane protein remains unclear.

Mn(II) Transport by Pneumococcus. Pneumococcal PsaA (34.6 kDa) has been crystallized in the apo, Mn(II)-bound, and Zn(II)-bound forms (Figure 4A–C).[46,58,61] 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.[62,63] The overall fold of PsaA is very similar in the three 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.[58] Zn(II) and Mn(II) are both coordinated by His67, His139, Glu205, and Asp280 (Table 2 and Figure 4B,C).[46,61] 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 chelates Zn(II) in a tetrahedral geometry, with Glu205 and Asp280 each coordinating Zn(II) in a monodentate manner (Table 2). 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;[46] however, Glu205 and/or Asp280 may afford bidentate interactions (Table 2). 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.

Despite the significance of *S. pneumoniae* in clinical settings, the consequences of host-mediated Mn(II) deprivation for this pathogen are largely unexplored. Two independent *in vitro* investigations revealed that CP inhibits the growth of *S.*

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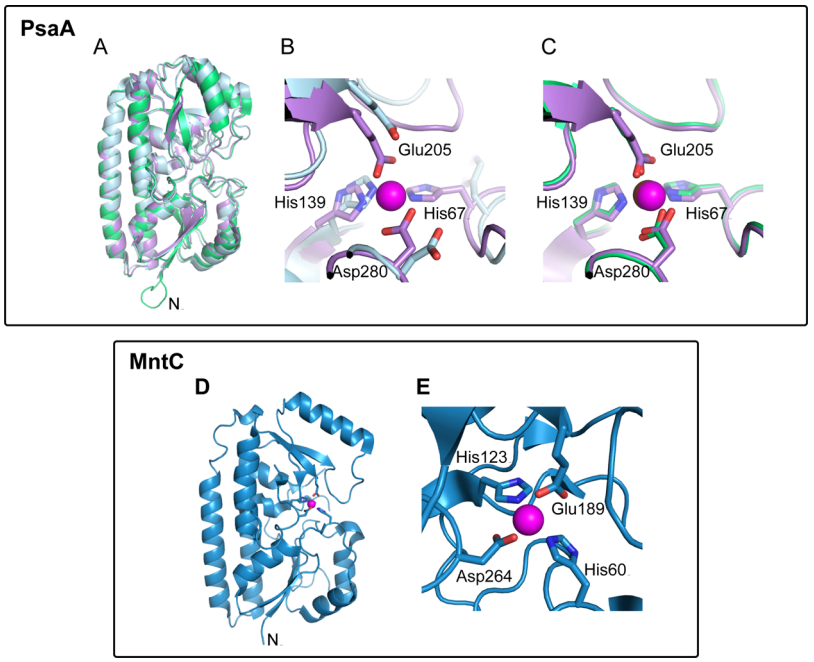

Figure 4. Crystal structures PsaA (A–C) and MntC (D, E). (A) Overlay of apo PsaA (light blue), Mn(II)–PsaA (lavender), and Zn(II)–PsaA (green). The metal ions are omitted from the overlay. (B, C) Overlays of the Mn(II)-binding site of PsaA with apo-PsaA (blue) and the Zn(II)-binding site of Zn(II)–PsaA (green). The Mn(II) ion from Mn(II)–PsaA is shown as a pink sphere. Apo PsaA, PDB: 3ZK7;[58] Mn(II)–PsaA, PDB: 3ZTT;[46] Zn(II)–PsaA, PDB: 1PSZ.[61] (D) The Mn(II)-bound form of *S. aureus* MntC. (E) An expansion of the Mn(II)-binding site. PDB: 4K3V.[45]

Table 2. Metal–Ligand Bond Distances in Selected Mn(II) Solute Binding Proteins

protein	residue/coordinating atom	metal–ligand bond distance (Å)
Mn(II)–PsaA[a,b]	His67/Nε2	2.1
	His139/Nε2	2.1
	Glu205/Oε1	2.1
	Glu205/Oε2	2.4
	Asp280/Oδ1	2.1
	Asp280/Oδ2	2.4
Zn(II)–PsaA[a,c]	His67/Nε2	2.0
	His139/Nε2	2.0
	Glu205/Oε1	2.0
	Glu205/Oε2	2.6
	Asp280/Oδ1	2.0
	Asp280/Oδ2	2.8
Mn(II)–MntC[d,e]	His50/Nε2	2.1
	His123/Nε2	2.1
	Glu189/Oε1	2.3
	Glu189/Oε2	2.8
	Asp264/Oδ1	2.2
	Asp264/Oδ2	2.3

[a]Numbering corresponds to the full-length PsaA. [b]PDB: 3ZTT. [c]PDB: 1PSZ. [d]Numbering corresponds to the soluble construct of MntC. [e]PDB: 4K3V.

pneumoniae.[2,64] In contrast, an animal model study of pneumonoccal infection reported attenuated pathogenesis in CP knockout mice relative to that in wild-type mice.[64] On the basis of the coordination chemistry observed for PsaA, a model whereby Zn(II) sequestration by CP prevents Zn(II) from inhibiting Mn(II) uptake was proposed.[64]

Mn(II) Transport by *Staphylococcus*. 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*.[49,65] MntC was recently crystallized with a putative Mn(II) ion in the metal-binding pocket (Figure 4D,E).[45] The Mn(II)-binding site is similar to that of PsaA and formed by His50, His123, Glu189, and Asp264 (Figure 4 and Table 2); however, the Mn(II) center is five-coordinate. Asp264 provides a bidentate ligand, whereas a monodentate interaction is observed for Glu189. ITC experiments performed in the presence of the Mn(II) competitor citrate revealed that MntC binds Mn(II) with nanomolar affinity (K_d = 4.4 ± 0.9 nM at 25 °C and pH 6.0).[45] MntC also coordinates Zn(II) and Cd(II). In contrast to Mn(II), binding of these d^{10} metal ions appears to be irreversible and may render the transporter inactive.[45] The irreversibility of Zn(II) binding is similar to the behavior reported for PsaA[58] and indicates that elevated levels of Zn(II) may also shut down the MntABC transporter.

CP exhibits growth inhibitory activity against *S. aureus*, and the MntABC and MntH transportation systems enable *in vitro* growth of *S. aureus* in the presence of CP.[66] Although the transcription of *mntA* and *mntH* increases 10- to 10 000-fold in the presence of inhibitory concentrations of CP,[66] strains of *S. aureus* lacking *mntA*, *mntB*, *mntC*, or *mntH* do not exhibit increased sensitivity to CP *in vitro*.[11,66] Rather, inactivation of both MntABC and MntH (Δ*mntC*Δ*mntH* strain) is required to increase the susceptibility of *S. aureus* to CP.[66] These results indicate that both transport machineries compete with CP for bioavailable Mn(II). Furthermore, redundancy of Mn(II)-uptake systems may be necessary for *S. aureus* to acquire sufficient quantities of Mn(II) at sites of infection where CP is abundant.

MICROBIAL MANGANESE ENZYMES CONTRIBUTE TO PATHOGENESIS

Identifying the manganese-dependent microbial processes that CP disrupts is of significant current interest. Following cellular

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uptake, manganese becomes incorporated into biomolecules. Microbial enzymes that employ manganese as a cofactor contribute to defense against oxidative stress, deoxynucleotide biosynthesis, primary metabolism, and antibiotic resistance (Figure 5). Recent investigations have focused on how CP modulates oxidative killing of bacteria as a result of Mn(II) chelation,[26,30,66] and additional Mn(II)-dependent microbial processes warrant consideration in future work.

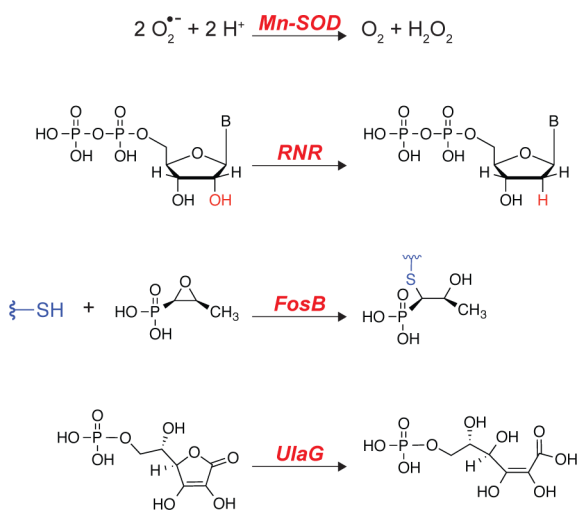

Figure 5. Examples of enzymes that utilize Mn as a cofactor. Mn-SOD disproportionates the superoxide radical, RNR converts ribonucleotides to deoxyribonucleotides, FosB inactivates the antibiotic fosfomycin, and UlaG hydrolyzes L-ascorbate-6-phosphate.

Superoxide Dismutases. 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.[67] The Fe, Mn, and Cu/Zn forms have been identified in human pathogens. Recent investigations addressing how CP influences bacterial susceptibility to oxidative stress have focused on *S. aureus*.[11,26,30,66] *S. aureus* strains produce two SODs, SodA and SodM.[68,69] SodA and SodM both exhibit a His_3Asp 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.[70−72] Given the expression of these two SODs, *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 *in vitro* and in animal models of *S. aureus* infection. Although not rigorously established to occur in *S. aureus*, small-molecule Mn complexes also disproportionate superoxide and provide protection against oxidative stress *in vivo*.[73,74]

Treatment of *S. aureus* Newman with CP *in vitro* reduces the total SOD activity of whole-cell lysates.[30] This effect was not observed upon mutation of sites 1 and 2 of CP, indicating that the transition metal-ion binding sites are necessary.[30] Moreover, the *S. aureus* SOD activity was restored upon addition of exogenous Mn(II) to the growth medium.[30] CP also increased the susceptibility of wild-type *S. aureus* to the superoxide-generating agent paraquat, and this effect was more pronounced for a Δ*sodA*Δ*sodM* knockout mutant of Newman.[30] Thus, SodA and SodM contribute to the protection of *S. aureus* against superoxide, and CP decreases this effect.[30] Subsequent studies revealed that site 2 (His_6) was required to attenuate the SOD activity of *S. aureus*.[26] Furthermore, 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 the mouse model of disease.[66] Taken together, these results indicate that site 2 of CP competes with bacterial metal-ion transporters for Mn(II) and that 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 assist the bactericidal oxidative burst by reducing SOD activity. Despite these observations from *in vitro* studies, the Δ*sodA*Δ*sodM* mutant in *S. aureus* was able to colonize both wild-type and CP knockout mice,[30] indicating that SODs may not be 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 requirement for iron and encodes a single Mn(II)-containing SOD.[75−77] Other virulent species that utilize Mn-SOD include *Neisseria gonorrheae*,[73] *Beauveria bassiana*,[78] *Streptococcus mutans*,[79] *Saccharomyces cerevisiae*,[80] *E. coli*,[81,82] *Haemophilus influenzae*,[83] and *Treponema pallidum*.[84] Many pathogens also express FeSOD and/or CuZnSOD.[67,85,86] Whether CP influences the (mis)metalation 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.

Ribonucleotide Reductase. Ribonucleotide reductases (RNRs) are essential enzymes that convert ribonucleotides to deoxyribonucleotides, the latter of which are the building blocks of DNA. Class I RNRs are metalloenzymes that contain dinuclear active sites. Characterized class Ib RNRs are active with a dinuclear Mn cofactor and therefore constitute compelling candidates for bacterial enzymes that are disrupted by CP-dependent Mn(II) sequestration.[87−91]

One example of a bacterial pathogen that expresses class Ib RNR is *Streptococcus sanguinis*, a causative agent of infective endocarditis.[92] A series of recent studies established that both manganese and class Ib RNR are important for *S. sanguinis* pathogenicity.[92−95] Deletion of the Mn(II) solute-binding protein SsaB (Δ*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 >1000-fold.[94] Subsequently, a *S. sanguinis* Δ*sodA* mutant was found to be less virulent than wild-type by only 10−100-fold. Taken together, these results suggested that other Mn-dependent processes contribute to heart valve colonization by *S. sanguinis*. The *S. sanguinis* genome encodes two forms of RNR, class Ib (*nrdHEKF* and *nrdI*) and class III (*nrdD*).[93] Because NrdD is a strictly anaerobic form of RNR, the class Ib enzyme is required for aerobic growth of *S. sanguinis*. Biochemical studies revealed that *S. sanguinis* class Ib RNR can be reconstituted with a diiron or dimanganese cofactor.[92] The latter form exhibits greater activity, utilizes a dimanganese(III) tyrosyl radical cofactor, and is postulated to be relevant *in vivo*.[92]

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In order to evaluate the contribution of the class Ib RNR to *S. sanguinis* virulence, the same rabbit model of infective endocarditis was employed,[93] and rabbits were infected with wild-type *S. sanguinis* or mutants lacking functional class Ib RNR.[93] In contrast to the parent strain, which was recovered from heart tissue as expected, the Δ*nrdHEKF* and Δ*nrdI* mutant strains were unable to survive *in vivo*.[93] Moreover, mutation of the class III RNR (Δ*nrdD*) had no effect on heart valve colonization.[93] These results demonstrated that manganese enzymes other than Mn-SOD contribute to virulence and revealed that disruption of RNR activity has a pronounced effect on *S. sanguinis* viability *in vivo*. Reduction of microbial 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 may also be considered from the standpoint of Mn(II) sequestration by the host. The sexually transmitted intracellular pathogen *Chlamydia trachomatis* expresses a class Ic RNR, and *in vitro* studies demonstrated that this enzyme is active with a bimetallic Mn/Fe cofactor.[96,97] It will be interesting to decipher whether Mn sequestration by the host perturbs its assembly. It should be noted that, 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*,[91] do not have genes encoding RNRs. Rather, these unusual microorganisms may scavenge deoxyribonucleotides from the host.

Other Mn Enzymes. There are many other enzymes that utilize Mn as a cofactor (Figure 5).[1] For instance, *S. aureus* FosB is a Mn(II)-dependent enzyme that inactivates the antibiotic fosfomycin.[98,99] Many enzymes involved in general metabolism, including *S.* Typhimurium propionate kinase and the *E. coli* lactonase UlaG, are active with added Mn(II).[100,101] A potential mycobacterial virulence factor and putative oxidase named Rv0223 requires a Mn/Fe cofactor for activity.[102] *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 2-fold increase in sphingomyelinase activity.[103] 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.[104] The consequences of metal chelation by CP on the growth of commensal bacteria is largely unexplored; Mn(II) and Zn(II) sequestration may also inhibit the growth of beneficial flora in the inflamed gut.[24,105,106]

SUMMARY AND OUTLOOK

The battle between host and pathogen for transition metal ions is an important facet of infectious disease. Manganese and CP are two intriguing players in this arena, and CP is currently the only known Mn(II)-sequestering host-defense protein in mammals. In recent years, chemical and biological initiatives have addressed the Mn(II)-binding properties of human CP as well as how CP affects select Mn(II)-dependent microbial processes and contributes to microbial infection. This work provides a foundation for future investigations pertaining to how CP contributes to metal-ion homeostasis at the host–pathogen interface and in other biological contexts. Further studies of this complex problem require the tools of chemistry and biology. Many different species of metal-bound CP exist, and the M(II)–CP speciation will be dependent on the metal-ion availability in any physiological environment. How CP modulates the ratios of bioavailable metal ions *in vivo*, and how microbes as well as the host respond to these changing nutrient levels, warrants rigorous evaluation. Because metabolic metal-ion requirements vary from microbe to microbe and because pathogens employ strain-specific strategies to evade the metal-withholding response, the effect of CP on microbial growth *in vitro* and *in vivo* must be considered on a case-by-case basis. Such initiatives will illuminate the interplay between CP and microbial virulence factors.

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Notes

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NOTE ADDED IN PROOF

While this manuscript was in review, a crystal structure of the NRAMP family member ScaDMT from *Staphylococcus capitis* was reported.[107]

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