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Lead(II) Binding in Natural and Artificial Proteins

Virginia Cangelosi¹, Leela Ruckthong², and Vincent L. Pecoraro²

¹Department of Chemistry, Michigan State University, 578 S. Shaw Ln., East Lansing, MI 48824-1322, USA

²Department of Chemistry, University of Michigan, 930 N. University, Ann Arbor, MI 48109-1055, USA

Abstract

This article describes recent attempts to understand the biological chemistry of lead using a synthetic biology approach. Lead binds to a variety of different biomolecules ranging from enzymes and regulatory and signaling proteins to bone matrix. We have focused on the interactions of this element in thiolate-rich sites that are found in metalloregulatory proteins such as Pbr, Znt, and CadC and in enzymes such as δ -aminolevulinic acid dehydratase (ALAD). In these proteins, Pb(II) is often found as a homoleptic and hemidirectic Pb(II) (SR)₃⁻ complex. Using first principles of biophysics, we have developed relatively short peptides that can associate into three-stranded coiled coils (3SCCs), in which a cysteine group is incorporated into the hydrophobic core to generate a (cysteine)₃ binding site. We describe how lead may be sequestered into these sites, the characteristic spectral features may be observed for such systems and we provide crystallographic insight on metal binding. The Pb(II) (SR)₃⁻ that is revealed within these α -helical assemblies forms a trigonal pyramidal structure (having an *endo* orientation) with distinct conformations than are also found in natural proteins (having an *exo* conformation). This structural insight, combined with ²⁰⁷Pb NMR spectroscopy, suggests that while Pb(II) prefers hemidirected Pb(II) (SR)₃⁻ scaffolds regardless of the protein fold, the way this is achieved within α -helical systems is different than in β -sheet or loop regions of proteins. These interactions between metal coordination preference and protein structural preference undoubtedly are exploited in natural systems to allow for protein conformation changes that define function. Thus, using a design approach that separates the numerous factors that lead to stable natural proteins allows us to extract fundamental concepts on how metals behave in biological systems.

Keywords

lead; lead toxicity; metalloregulatory proteins; ²⁰⁷Pb NMR; protein design

1. INTRODUCTION

Throughout history, lead (Pb) has been both an instrument for, and a hindrance to, human progress. It's been used as a wine preservative, in cosmetics, in cookware, in printing presses, in paint, and more [1, 2]. Utilizing tetraethyl lead as an antiknock gasoline additive in the 1920s improved the performance of engines, but it also introduced lead into the environment in unprecedented amounts. Since lead additives were phased out of gasoline,

the average blood lead level (BLL) of children in the United States dropped significantly from 15.0 $\mu\text{g}/\text{dL}$ between 1976–1980 to 1.84 $\mu\text{g}/\text{dL}$ in 2009–2010 [3, 4]. The malleability of lead allowed for the first plumbing systems, yet contaminated drinking water from lead pipes and soldering is a problem that still haunts us today. At the time this chapter was written, the entire city of Flint, Michigan (population 100,000) in the United States is being advised to drink bottled water because of the contamination of the local water supply [5]. The city switched water sources in an attempt to save money, but failed to treat the new water with corrosion inhibitors, resulting in the leaching of lead from pipes. The Environmental Protection Agency (EPA) sets an acceptable threshold of 15 parts-per-billion (ppb) for drinking water, yet lead levels of over 1000 ppb were measured in water coming straight from the taps of some residents' homes. The problem was discovered by a doctor who noticed a doubling in the number of children with elevated BLLs (above 5 $\mu\text{g}/\text{dL}$) [6].

Unfortunately these children are already starting to experience the health problems associated with lead exposure. Lead causes a barrage of health problems including anemia, renal impairment, hypertension, infertility, violent behavior, decreased intelligence, and more [4, 7–11]. Lead causes developmental problems in the fetuses and, even if a pregnant mother is not exposed to a new source, lead stored in the bones of women can be mobilized during pregnancy and lactation [12–14]. A recent study shows that the epigenetic consequences are even farther reaching than that. Lead exposure while pregnant affects the DNA methylation status of the fetal germ cells in not just her child, but her grandchildren [15]. The diversity of lead-related health problems result from lead's ability to bind to, and disrupt, the function of a multitude of biomolecules.

Many of the health problems caused by lead can be explained by studying the natural proteins to which it binds. The coordination of lead to δ -aminolevulinic acid dehydratase (ALAD) interrupts the biosynthesis of heme, causing anemia [16]. Memory and growth issues can result from lead coordinating to calcium-binding proteins [17]. Many of the neurological problems associated with lead exposure could result from Pb(II) coordination to zinc finger proteins [18]. There is still much to discover about how Pb(II) behaves in the body and one way to learn more is to study proteins that appear to have evolved specifically for Pb(II) coordination. One of the difficulties in studying natural proteins is that they are large and often difficult to isolate and characterize due to the specific conditions they require for folding. A way around this problem is to use simplified proteins, designed from scratch, to coordinate lead in its preferred geometry. In this chapter we will discuss the application of *de novo* protein design to lead binding and the characterization of these proteins by spectroscopic techniques including ^{207}Pb nuclear magnetic resonance spectroscopy (^{207}Pb NMR).

2. LEAD IN NATURAL SYSTEMS

The size, thiophilicity, and ability to bind many types of ligands in many coordination geometries makes Pb(II) a particularly problematic ion in biological systems. In many proteins and biomolecules it is able to replace physiologically relevant ions, but due to differences in chemistry, cannot carry out their functions. Lead complexes are kinetically labile and thermodynamically stable under aqueous conditions [19, 20]. In proteins, Pb(II) is

coordinated by sulfur, oxygen, and nitrogen donors. Although coordination by cysteine (Cys) is preferred over glutamic acid (Glu) or histidine (His), mixed donor ligand environments, such as those found in many proteins, are harder to predict [19]. In a 2008 analysis of 48 lead-containing crystal structures in the Protein Data Bank, replacement of a native metal ion by lead was observed in one-third of the lead-containing sites [17]. The other two-thirds of the lead ions were coordinated to proteins opportunistically in sites that did not previously contain metal ions. Lead ions were observed with up to 9 ligands, but 2–5 were observed in three-quarters of the sites. In these structures, 59 % of the ligands were glutamate and aspartate side chains and another 20 % were water molecules. Side chain nitrogen atoms made up 5 % of the ligands whereas side chain and small molecule sulfur made up only 7 %. This may seem surprising considering the thiophilicity of lead, but it is impossible to determine which of these sites are relevant *in vivo* and which result from the crystallization process. Still, the analysis provides useful information on the diversity of ligands and coordination environments that are possible for lead within proteins (Figure 1).

Many of the best-studied natural protein systems described below do contain Pb(II) coordinated in Cys₃ environments, including the metalloregulatory proteins that appear to have evolved to control Pb(II) concentrations in cells (Section 2.5). One of the most-studied targets for Pb(II) toxicity, δ -aminolevulinic acid dehydratase contains a Zn(II)Cys₃ site that is substituted with Pb(II) (Section 2.2). Pb(II) also substitutes for Zn(II) in zinc finger proteins, with the affinity for this ion correlated with the total number of Cys residues at the metal binding site. Thus, zinc finger proteins containing three or more Cys are best able to bind Pb(II), with the canonical His₂Cys₂ having lower affinity for this protein class. Unfortunately, Pb(II) is not a functional substitute for Zn(II) (Section 2.4 below) in any of these systems, in part because it is believed not to bind well to histidine. Pb(II)'s radius (1.19 Å) is significantly larger than that of Zn(II) (0.74 Å), it is less acidic, and it has different coordination preferences. The Pb–S bond distances for Pb(II)Cys₃ sites are all between 2.6 and 2.8 Å [18, 21–24].

2.1. Glutathione and Other Small Peptides

In cells, glutathione (GSH) and thiol-rich proteins may act as a natural “buffering system” for lead *in vivo* [20]. GSH, which has concentrations of greater than 1 mM in most mammalian cells, coordinates toxic metal ions and transfers them to proteins, providing some protection against their toxic effects [25–27]. The cysteine-rich metallothioneins and phytochelatins are known to bind lead that has been transferred from lead complexes of GSH *in vitro* [25]. In humans, GSH likely complexes Pb(II) *in vivo*. This is supported by the observation that humans with work-related exposure to lead have decreased GSH levels and decreased GSH-dependent enzyme activities [28, 29]. GSH, which contains multiple potential donors, coordinates lead using its sulfur donor with additional minor interactions with the C-terminal carboxylate. In line with lead's coordination preferences discussed above, [Pb(II)(GS)₃][−] is the predominately formed species in solution with Pb–S distances of 2.65 Å. (Figure 2) [22, 30–33].

2.2. δ -Aminolevulinic Acid Dehydratase

In humans, the metalloenzyme δ -aminolevulinic acid dehydratase (ALAD), also known as porphobilinogen synthase) carries out the second step of heme biosynthesis. The condensation of two molecules of δ -aminolevulinic acid (ALA) to produce porphobilinogen, a precursor of heme, is catalyzed by a Zn(II)-Cys₃ site in ALAD. Inhibition of this enzyme process leads to hemoglobin deficiency which causes anemia. Pb(II), which is estimated to have a 25-fold greater affinity than Zn(II) for the active site of ALAD, can replace the Zn(II) ion and inhibit activity [34]. This occurs at concentrations as low as femtomolar lead ($K_i = 0.07$ pM) [34]. The consequences of inhibition are two-fold: not only is heme synthesis halted, but neurotoxic ALA accumulates in the system [16, 35].

The active site of ALAD contains a Zn(II) ion coordinated by three Cys residues and one exogenous water molecule in a pseudo-tetrahedral coordination geometry. Thiophilic Pb(II) replaces the Zn(II) ion with minimal disruption to the fold of the protein or the structure of the active site [16, 21]. The crystal structure of Pb(II)-ALAD shows the Pb(II) ion coordinated in a trigonal pyramidal, hemidirected manner geometry to the Cys₃ site (Figure 3). Although the coordination geometries of the Zn(II) and Pb(II) ions to the endogenous protein ligands are similar, they are not identical and the chemistry of these ions is very different [21, 36]. Coordination of ALA to Zn(II) allows activation of the ketone group, which is essential to the reaction mechanism. Pb(II), which is a weaker Lewis acid than Zn(II), cannot activate ALA [37]. Additionally, the Pb–S bonds are an average of 0.54 Å longer than the Zn–S bonds, causing Pb(II) to protrude farther in to the substrate-binding cavity [16, 21]. Most significantly, Pb(II)'s lone pair of electrons blocks the incoming ALA substrate from binding at the required angle^[37]. Finally, a normally non-bonding serine residue (Ser 179) is 0.9 Å closer to the metal ion in the lead structure than in the zinc structure [16, 21].

In addition to coordinating and inhibiting the active site of ALAD, Pb(II) ions can bind opportunistically (or non-specifically) to other parts of the protein. The crystal structure of lead-coordinated ALAD reveals a second ion bound within the substrate-binding pocket with a Pb–Pb distance of 4.4 Å [16, 21]. This nonspecific binding increases the possibility of increased Pb(II) solubility and transport [17].

In erythrocytes, the majority of lead coordination occurs within ALAD. Workers who are occupationally exposed to lead often have elevated blood lead levels (BLLs). In samples of their blood, between 35 and 81 % of the detected lead was bound to ALAD [38]. In whole blood, ALAD was found to have a lead-binding capacity of ~40 µg/dL or 1.9 µM [38]. Each ALAD octamer was coordinated to an average of ~6 Pb(II) ions. Despite this being less than one ion per monomer, ALAD activity is greatly reduced. At BLLs of µg/dL (720 nM) there are only 1–2 bound lead atoms per octamer, yet ALAD activity is decreased by 50 % [38].

2.3. Calmodulin

Another family of metalloproteins that are known to be disrupted by lead are the calcium-binding proteins (CaBPs). CaBPs are signal transducers that use Ca(II) ions to govern cellular activities. One of the most heavily studied families of CaBPs is calmodulin (CaM)

[39]. CaM is found in all eukaryotic cells and plays a role in the calcium signaling pathways for more than 100 biological processes. These include memory, growth, inflammation, immune response, and muscle contraction. Coordination and release of Ca(II) ions causes structural changes within CaM. This serves as an on/off switch for CaM to bind to and activate other proteins [40]. Calcium binding occurs to oxygen atoms from side chain and backbone residues within four highly conserved helix-loop-helix domains known as EF hand motifs [39]. Similar EF hand motifs are found in over half of all CaBPs, so CaM is prototypical for this class of proteins.

The four EF hand motifs in CaM can also bind Pb(II) ions and do so simultaneously and with high affinity [41, 42]. Crystal structures of calcium- and lead-bound CaM show that these two ions have almost identical coordinations and very similar occupancies within the metal-binding site (Figure 4) [43, 44]. Lead-for-calcium ion replacement causes minimal perturbation within the metal-binding sites and is not expected to cause major conformational changes to CaM. While the C-terminal domains of Ca(II)- and Pb(II)-bound CaM show close structural agreement, the N-terminal domain of Pb(II)-CaM participates in extensive Pb(II)-mediated contact with neighboring molecules and shows significant structural differences [44]. It is proposed that the changes in structure are due to the opportunistic coordination of 10 additional Pb(II) ions outside of the metal binding sites to the protein's surface (Figure 5). The conditions required for crystal growth of metalloproteins often involve high concentrations of metal ions, making surface binding a common phenomenon during crystallization and not necessarily representative of *in vivo* metal binding. In this case the surface binding occurs in areas of high electrostatic potential leading to the proposal that this high affinity binding causes conformational changes to the protein's structure [17, 44].

Since these structural changes were observed *in vitro*, it is unclear what, if any, the effects of opportunistic Pb(II)-binding are on CaM *in vivo* or if it even occurs. It has been observed *in vitro* that in the presence of physiological concentrations of free Ca(II), CaM can be activated by Pb(II) at concentrations below 50 pM [45]. While lead is normally thought to inhibit protein function, this suggests that very low concentrations of Pb(II) may actually amplify intracellular Ca(II) signaling thus stimulating other cellular processes. This is further supported by the stimulatory effects of low concentrations of Pb(II) on the activation of myosin light-chain kinase by Pb(II)-CaM [46].

Lead ions coordinate to CaM with a higher affinity than Ca(II) [47]. Coordination at all four of the Ca(II)-binding sites within CaM has been observed, but with unequal affinities to the different sites [41, 48]. In the metal binding sites within the C-terminal domain, Pb(II) binds with a 3-fold higher affinity than Ca(II): $K_D = 0.73 \mu\text{M}$ for Pb(II) and $K_D = 2.0 \mu\text{M}$ for Ca(II). In the N-terminal domain, there is an 8-fold preference for Pb(II): $K_D = 1.4 \mu\text{M}$ for Pb(II) and $K_D = 11.5 \mu\text{M}$ for Ca(II) [47]. This is consistent with observations of other CaBPs that coordinate Pb(II) with higher affinity than Ca(II). The measured dissociation constant for Pb(II) ($6.2 \times 10^{-8} \text{ M}$) is lower than that for Ca(II) ($5 \times 10^{-7} \text{ M}$) in bovine and chick intestinal CaBPs, indicating stronger lead binding [42]. In each case, the Pb(II) ions appear to bind directly to the calcium-binding sites. It should be noted, however, that these studies were carried out *in vitro* and the comparison *in vivo* is unknown.

2.4. Zinc Finger Proteins

Zinc finger proteins are one of the most abundant protein types in eukaryotic cells and have an extremely diverse set of roles. Best known as transcriptional regulators, zinc fingers are important for DNA recognition, transcriptional activation, mediation of protein-protein interactions, RNA packaging, and much more [49]. Disruption of these processes in children would affect neurological development. Broadly defined, a zinc finger is a small protein domain that requires one or more Zn(II) ions for stabilization of the fold [50]. The classic zinc finger has a $\beta\beta\alpha$ structure that binds zinc ions in a tetrahedral geometry with four ligands composed of mixtures of Cys and His (two from within the turn of the β -hairpin and two from the C-terminal end of the α -helix). Residues on the exterior of the zinc finger (α -helix side chains) recognize and bind to specific DNA, RNA, or protein sequences.

Lead can cause misfolding when it interacts with zinc finger proteins in cell nuclei and the cytosol [51–53]. *In vitro* competition experiments with zinc finger consensus peptides show that Zn(II) and Pb(II) equilibrate rapidly [20]. Lead speciation appears to be under thermodynamic control and the relative binding affinities of the metals determine the ratio of the metals bound to the protein. This contradicts the previous assumption that lead is kinetically inert after binding non-specifically to cysteine-rich proteins. This suggests that under physiological conditions, Pb(II) should be able to compete effectively with Zn(II) for binding to cysteine-rich sites. Indeed, this is observed in both artificial and natural zinc finger proteins.

Even though four potentially chelating residues are present, only three Cys residues actually bind Pb(II) within Cys₄ and Cys₃His zinc fingers, resulting in a misfolding of the protein [18, 33, 54]. The number and positions of Cys and His residues within the zinc finger proteins affects the affinities of both Zn(II) and Pb(II) (Figure 6) [18, 20, 55]. Zinc finger consensus peptides containing four Cys residues (CP-CCCC) bind both Zn(II) and Pb(II) more strongly than those with containing only two or three Cys residues (Table 1). For lead, CP-CCCC binds about three orders of magnitude more strongly than the other peptides. The position of the Cys residues is also important. Although both contain one His and three Cys residues, Zn(II) binds an order of magnitude less strongly to CP-CCCH than it binds to CP-CCHC [18]. However, Pb(II) binds to CP-CCCH almost an order of magnitude *more* strongly than CP-CCHC. Taken together, there is a significant difference in the competitiveness of these two peptides for lead based on the positions of the coordinating residues. This is likely because the first two cysteines are separated by only a proline residue and they are too close together to coordinate Pb(II) in a trigonal pyramidal geometry [18]. A peptide with CP-XCCC is predicted to form the most stable complex with lead because of the spacing of the Cys residues. Despite the fact that CP-CCCC only uses three Cys residues to do so, it coordinates Pb(II) 30-fold more strongly than Zn(II). Considering physiological concentrations and the rapid equilibration of these sites, this difference means that lead should be able to compete with zinc binding in cells [20, 56].

While most of the quantitative data on lead's interaction with zinc fingers comes from experiments with synthetic consensus peptides, natural zinc fingers are also known to bind Pb(II). One specific type of protein that binds GATA sequences within DNA using classic zinc fingers are GATA proteins. These transcription factors are critical for development of

blood cells, the central nervous system, the genitourinary system, and cardiac health. Lead can displace the zinc ions within the Cys₄ binding sites within GATA proteins resulting in those proteins having a decreased DNA-binding affinity and a decreased ability to activate transcription [57]. Canonical zinc fingers containing Cys₂His₂ sites may also be targets. The transcription factors TFIIIA and Sp1 have inhibited DNA binding in the presence of micromolar lead, although Pb(II) may not compete effectively with Zn(II) for Cys₂His₂ sites *in vivo* [20, 58, 59]. The human protamine HP2 is inhibited upon binding Pb(II) at two different sites, possibly explaining the decreased fertility of men occupationally exposed to lead [60].

2.5. Metalloregulatory Proteins

Bacteria living in metal-rich environments have evolved defenses against the toxic effects of metals that range from storing them in inert forms within the cell to expelling them. Concentrations within the cell of purely toxic metals such as mercury, arsenic, cadmium, and lead as well as physiologically-necessary metals that are toxic in excess must be controlled [61–63].

2.5.1. The Lead Resistance Operon *pbr*—Some bacteria have evolved defenses for dealing with high concentrations of lead in the environment. The complex set of genes, *pbrRTUABCD*, encode lead resistance in the bacterium *C. metallidurans* strain CH34 [64]. Compared to the metalloregulation carried out by *znt* and *cad* (see below), the *pbr* operon is relatively complex (Figure 7). It is encoded for the regulatory protein PbrR, the uptake protein PbrT, the efflux ATPase PbrA, the inner-membrane fusion phosphatase PbrB/PbrC, and a lead-binding protein PbrD [64–66]. There may also be an inner membrane permease gene (*pbrU*) present [66, 67]. Regulation of lead starts with transport of Pb(II) into the cell by the uptake protein PbrT in order to protect the cell's exterior. Once within the cell, it is picked up by the metallochaperone PbrD and transported to the efflux ATPase PbrA (or ZntA) which exports it from the cytosol to the periplasm. In the periplasm, PbrB/PbrC produce phosphates that precipitate Pb(II), sequestering the ion from circulation [65]. On top of removing harmful Pb(II) ions from the external environment and storing them as inert lead phosphates within the cell, this process also protects *C. metallidurans* strain CH34 by increasing the pH around the external environment of the cells. This results in the precipitation of lead in the local cell environment as lead hydroxide and lead carbonate which lowers the local concentration of Pb(II) [64]. Protecting cells in this manner is not a completely unknown phenomenon; the cadmium-zinc-cobalt resistance system *czc* protects *C. metallidurans* in a similar manner [68].

Transcription of the *pbrRTUABCD* genes is controlled by the lead regulatory protein PbrR, which is a member of the MerR family of metal-sensing regulatory proteins. PbrR is a homodimeric protein that binds to the promoter region of the *pbr* operon in the absence of lead [69]. When lead is present, it is thought to bind to three cysteine residues (Cys14, Cys79, and Cys134) in the protein, one of which is found in the helix-turn-helix DNA binding domain (Cys14). Binding changes the conformation of the protein and, as the concentration of Pb(II) increases, the affinity of PbrR for the promoter region decreases.

Changes to the conformation of the DNA strand makes the area accessible to RNA polymerase and transcription of the genes follows [69].

Binding studies were carried out with a PbrR homologue, PbrR691, which is also found in *C. metallidurans* strain CH34. Each dimer binds one equivalent of Pb(II) with about 1000-fold selectivity over other divalent metals (Zn(II), Cu(II), Ni(II), Co(II), Hg(II), and Cd(II)) and a $K_D = 0.2 \mu\text{M}$ [70]. In fact, this protein is so selective for Pb(II) that it was developed into a Pb(II) sensor [70]. The significant selectivity may come from the binding site being preorganized to coordinate Pb(II) in a hemidirected geometry, which is lead's preference at low coordination numbers [23]. Lead is coordinated by cysteine residues, probably Cys78, Cys113, and Cys122, which are conserved when aligning the sequence with MerR [23]. Publication of the X-ray crystal structure of PbrR-691 is anticipated soon as an unpublished structure has been deposited in the RCSB Protein Data Bank (unreleased structure ID 5DBZ) [71, 72].

The *pbr* operon encodes two different transmembrane transport proteins, PbrA and PbrT. Although it is not an ATPase, the inner-membrane protein PbrT appears to be a Pb(II)-uptake protein [64]. This is supported by hypersensitivity of the cell to Pb(II) when PbrT is expressed in isolation from the remainder of the *pbr* operon [64]. Once within the cell, Pb(II) appears to be transported by the ~200 amino acid protein PbrD. This metallochaperone has the potential binding site(s) Cys-7X-Cys-Cys-7X-Cys-7X-His-14X-Cys and theoretically sequesters Pb(II), transporting ions to the efflux ATPases and lowering cytoplasmic concentrations of free lead. This is supported by the observations that cells lacking PbrD have higher cytoplasmic lead levels and have higher rates of induction of efflux proteins such as PbrA and ZntA [66]. The influx of Pb(II) by PbrT is counteracted by the efflux of Pb(II) by the PbrA. PbrA is an ATPase with similar properties to CadA and ZntA (see Sections 2.5.2 and 2.5.3) [65]. While most P-type ATPases that transport soft metals contain the consensus Cys-X-X-Cys sequence, this is replaced in PbrA by two Cys-Pro-Thr-Glu-Glu metal-binding sites that may contribute to the selectivity of this proteins for lead [64].

PbrR also controls the gene for the fusion protein PbrBC, an undecaprenyl pyrophosphate (C55-PP) phosphatase, found in the periplasm bound to the inner membrane [65–67]. Resistance appears to come from the *pbrB* portion of the gene, because removal of the *pbrC* portion does not affect cell viability in the presence of lead [65]. When PbrBC is expressed, there is an increased production of phosphates in the periplasm which precipitate Pb(II), although the exact chemical composition of the precipitate is unknown. PbrBC works within the greater lead resistance mechanism of *C. metallidurans* strain CH34. If Pb(II) cannot reach the periplasm due to the absence of P-type ATPase efflux pumps (PbrA and ZntA), there is an overproduction of phosphate in the periplasm and a buildup of lead in the cytoplasm [65].

2.5.2. The Zinc Resistance Operon *znt*—In *Escherichia coli*, Pb(II) can be removed from cells by the P-type ATPase ZntA [73, 74]. The native substrate for ZntA is Zn(II), which, despite being an essential metal, is toxic in excess. However, in the presence of Pb(II), Zn(II) transport is inhibited [75]. Pb(II) activates the ATPase, increasing the rate of phosphorylation and conferring resistance to Pb(II) [76]. The highest ATPase activities are

observed for Pb(II)-Cys and Pb(II)-GSH complexes, suggesting that thiolates may carry Pb(II) ions to ZntA and play an important role in the functioning of the enzyme *in vivo* [77]. This is supported by the fact that at a pH greater than 6, Cd(II) and Hg(II) inhibit ZntA activity while Cd(II)-thiolate and Hg(II)-thiolate complexes stimulate activity. Both of these metals have a higher affinity than Pb(II) or Zn(II) for ZntA and may form dead-end complexes upon coordinating Cys residues. However, the mechanism of binding, the exact binding mode of Pb(II), and the role of high affinity thiolate molecules are unclear.

ZntA expression is controlled by ZntR, a homodimeric member of the MerR family of transcriptional regulators that shares many characteristics with PbrR and responds to Zn(II), Cd(II), and Pb(II) binding [78, 79]. ZntR binds Zn(II) very strongly ($\log K_D = -14.8$ at pH 7.0), but the affinity of lead for the protein is unknown [80]. There is not yet an X-ray crystal structure showing Pb(II) bound to ZntR, but the structure of an N-terminally truncated Zn(II)-bound fragment of ZntR may give some insight. This structure reveals two metal-binding domains at the dimeric interface that are each bound to two tetrahedral Zn(II) ions [81]. Within each site, the ions are 3.6 Å apart and coordinated by two residues from one monomer (Cys114 and Cys124 for Zn1 and Cys115 and His119 for Zn2), a bridging Cys ligand from the other monomer (Cys79), and an oxygen atom from a bridging sulfate or phosphate ion (Figure 8). Neither Cys115 nor His119 are required for induction of ZntR by Pb(II), suggesting that Pb(II) could be coordinated in a trigonal pyramidal geometry by Cys79, Cys114, and Cys124 in a similar position to Zn1 [82]. How the differences in size and acidity of the metals are affecting the conformation and the function of the protein is unknown.

While the binding of Zn(II) and Pb(II) may be slightly different, once bound the mechanism by which ZntR induces gene expression is likely similar (and reminiscent of transcriptional regulation by MerR). Apo-ZntR binds the ZntA-promoter region and distorts the DNA by bending the strand toward itself, causing the formation of two kinks [83]. These kinks restrict access by RNA polymerase to the promoter site and transcription is repressed. Zn(II) coordination causes slight changes to the shape of ZntR, relaxing the bend in the DNA and unwinding the center of the promoter region. This allows RNA polymerase full access to the operator and transcription is activated.

2.5.3. The Cadmium Resistance Operon *cad*—The *cadCA* operon, consisting of the efflux ATPase CadA and the metalloregulatory protein CadC, provide heavy metal resistance to *Staphylococcus aureus* [84]. Homologous to ZntA, CadA is a P-type ATPase that exports Cd(II), Zn(II), and Pb(II) [75]. Unlike ZntR, the metalloregulatory CadC is a member of the ArsR/SmtB family of transcriptional regulators. In the absence of inducing cations, the homodimer CadC binds specifically to the *cad* operator on the DNA, suppressing transcription of CadA [85]. Upon binding to Pb(II), Cd(II), and Bi(III), CadC changes conformation and releases the DNA, allowing transcription of CadA. Complete derepression of *cadCA* occurs at lower concentrations of Pb(II) than Cd(II) (200 nM and 300 nM, respectively), suggesting that CadC is primarily a lead regulator [75].

The mechanism of transcriptional regulation is supported by the X-ray crystal structure of apo-CadC (1.9 Å resolution) (Figure 9) [86]. The putative DNA binding domains are helix-

turn-helix motifs composed of helices 4 and 5 (and 4' and 5'). The two metal binding sites, both of which are required for derepression, lie in the interface of the homodimer and are composed of two cysteine residues (Cys7 and Cys11) from helix 1' (and 1) and two (Cys58 and Cys60) from helix 4 (and 4') [87–90]. Within each site, all four Cys residues coordinate Cd(II) in a distorted tetrahedral geometry. An alternative idea is that this fourth thiolate ligand is easily displaced by water so that an equilibrium structure between $[\text{Cd}(\text{SR})_4]^{2-}$ and $[\text{Cd}(\text{SR})_3(\text{H}_2\text{O})]^-$ is the best description. This idea is supported by ^{113}Cd NMR spectroscopy and the notion that having a more dynamic metal binding site would facilitate the rapid kinetics necessary for a metal responsive switch [91]. Lead, which prefers Cys_3 coordination, does not require Cys11 for de-repression and instead forms a trigonal pyramidal $\text{Pb}(\text{II})\text{Cys}_3$ complex [92, 93]. Metallation of the sites moves helix 1' toward helix 4 (and helix 1 toward helix 4'), which sterically blocks a section of the DNA-binding motif causing the protein to release from the DNA.

Metal binding to CadC occurs in two distinct kinetic phases [94]. First, there is a bimolecular encounter between CadC and the metal ion. The rate of this step is metal-dependent ($\text{Pb}(\text{II}) > \text{Bi}(\text{III}) \gg \text{Co}(\text{II})$). Next, a slower isomerization step occurs which is rate-independent of the type of metal bound and appears to utilize Cys7.

3. LEAD CHEMISTRY WITH DESIGNED PROTEINS

Until very recently, detailed structural information regarding the active site structure of many of the heavy metal binding proteins described in the previous sections was not existent. Even as more clarity on the natural systems is being revealed, there are still issues dealing with the fundamental interaction of heavy metals with proteins that are often difficult to assess in these systems. As mentioned above, there has been significant debate about the first coordination sphere ligands that bind to Cd(II) in CadC, and whether there may be multiple species that can exist in order to control exchange kinetics for this metalloregulatory switch [91–94].

We have been particularly interested in defining how heavy metals will bind intrinsically to proteins, what are their coordination preferences and how well does structure dictate function in these systems. Unfortunately, the answers to such fundamental questions may be obscured by the complexity of natural systems, which have to perform functions beyond the binding of the target metal. Thus, we have sought a simplified construct that may directly address the issues of structural preference, site stability, exchange kinetics and site selectivity by using *de novo* designed proteins that allow interrogation of the heavy metal protein relationship in a more detailed and precise fashion. The following section presents our strategy for achieving these objectives with the biologically important Pb(II) cation.

3.1. Design Strategy for Binding Lead

Our strategy to prepare small proteins to bind lead effectively began after more general advances in protein design had been established [95]. One of the most important concepts we utilize is that of exclusion of hydrophobes from water or “hydrophobic collapse” [96–101]. The aggregation of the peptides that we synthesize is dominated by the favorable energetics of desolvation of hydrophobic residues such as leucine, isoleucine or valine when

the desired structure is formed. Our target scaffold was the three-stranded coiled coil motif (3SCC). To achieve such a structure, one should synthesize peptides that are amphipathic, meaning one side is hydrophobic and the opposite face is hydrophilic. In doing so, one can exploit the strong stabilization obtained from hydrophobic collapse, while generating an aggregate that is still water-soluble. Examination of the helical wheel diagram for a parallel 3SCC, shown as Figure 10, illustrates this point well. All of the hydrophobic residues are oriented toward the interior of the assembly, while hydrophilic residues point either to the helical interface, where they may form salt bridges with adjacent helices, or toward the solvent, providing for water solubility.

Of course, one must have a strategy to align these residues to adopt the desired positions shown in the helical wheel diagram. This is achieved by employing the concept of the “heptad repeat” [100, 102–105]. Seven amino acids, typically designated *abcdefg*, are repeated along the primary sequence of the desired peptide. The N- and C-terminal ends are capped with glycines that have been acetylated or amidated, respectively. These modifications to glycine induce the folding of the sequence into an α -helix and decrease the resultant helical dipole. Numerous studies have shown that a binary pattern, either using hydrophobic or hydrophilic residues, will lead to helical folding when one places these amino acids in the appropriate position of the heptad [106–110]. Thus, the *a* and *d* positions contain hydrophobes while *b*, *e*, *f* and *g* positions contain hydrophilic residues. The *c* position is neutral to hydrophilic/hydrophobic concerns and typically contains the helix inducing residue alanine.

The α -C positions in an α -helix rotate 100 degrees with respect to the previous amino acid in the sequence [111]. Thus, a heptad of amino acid rotates the α -C positions 700 degrees across its length. This is 20 degrees short of two full turns of the helix, therefore, to align residues in such a way to maximize hydrophobic contacts, the resultant α -helix must coil around the other helices forming the scaffold. It is this mismatch of rotation that leads to the supercoiling in these systems.

The typical sequence that we utilize is based on the heptad repeat LKALEEK [112]. Notice that the *a* and *d* positions contain the bulky hydrophobe leucine. Just as considered above, rotation from the *a* leucine to the *d* leucine requires 300 degrees, therefore, these residues do not lie directly above one another in the core of the 3SCC (Figure 11). Similarly, moving from the *d* leucine to the *a'* leucine (the first leucine in a subsequent heptad) requires 400 degrees, which is 40 degrees out of rotational phase. As will be demonstrated below, this rotational disparity has significant consequences for the binding of heavy metals. Another important consequence of the heptad repeat is that two residues intervene between the *a* and *d* layers, whereas three residues separate the *d* and *a* positions. This asymmetry results in different interlayer spacing of the hydrophobes, and subsequently, heavy metal binding ligands that will be placed within the hydrophobic interior.

The heptad orientation also has consequences for the amino acids in the hydrophilic positions. In this case, principles of “negative design” are utilized [102]. Typically, chemists think of building stable structures by minimizing energetic interactions; however, a negative design strategy attempts to generate a desired structure by destabilizing unwanted

interactions. For this reason, the residues, particularly in the *e* and *g* positions, are chosen to select against anti-parallel helical orientations [113]. The number of strands in a bundle (2SCC, 3SCC, 4SCC or higher) is controlled by the choice of hydrophobe (e.g., by its size) and the pattern of the chosen hydrophilic residues (charge, length) in the *e* and *g* positions [108, 114–117].

Once the heptad sequence is established, it is now necessary to determine the length of the peptide [118,119]. We have worked with three lengths containing three (Baby), four (TRI) or five (GRAND) heptads, which are generally referred to as the TRI family of peptides as these have been designed to assemble into parallel 3SCCs using the principles just described [120]. Representative sequences are presented in Table 2. The free energy of folding for these peptides increases as the length of the peptide increase [120]. Thus, GRAND peptides fold into 3SCCs at sub-nanomolar concentrations whereas the much shorter Baby peptides are only partially folded at micromolar levels. In addition to being important for allowing the preparation of stable aggregates to interrogate in solution, the added stability of GRAND peptides has been important for the growth of crystals using the related CoilSer peptides (CS or GRANDCoilSer, Table 2).

While this TRI family leads to very stable assemblies, they are not yet capable of high affinity and selective metal binding. To achieve this goal one must modify the sequence in order to incorporate a ligand that has a strong propensity to bind large, soft heavy metals such as Pb(II). The ligand of choice has been cysteine. A substitution of a hydrophobic residue either at *a* or *d* position allows for a three Cys binding site. The original designs were TRIL12C and TRL16C in which a Cys is replaced at the twelfth position (*d* site) and the sixteenth position (*a* site), respectively [24]. In solution studies, these Cys₃ binding sites were found to bind several types of heavy metal (e.g., Pb(II), Hg(II) Cd(II), As(III) and Bi(III)) [24,121–145]; however, only evidence of Pb(II) binding will be addressed in this chapter. Readers who are interested in Cd(II) and Hg(II) studies with these peptides are referred to other articles [146–149]. As will be demonstrated below, metal complexation at *a* sites is not necessarily equivalent to binding to *d* sites, which leads to interesting differences in structure, spectroscopy, and selectivity. While the vast majority of Pb(II) biological sites are substituted Ca(II) environments, complexes of lead with thiolate ligands will be emphasized in the following sections. This limitation is because most reported studies with designed proteins binding heavy metals have focused on soft metal complexation.

3.2. Lead Spectroscopic Features

The bioinorganic chemistry of Pb(II)-interactions with sulfurs can be followed by a variety of spectroscopic methods. Previously, there was a widespread misconception that the filled d-shell of lead yields spectroscopically silent species impeding attempts to study lead binding to proteins. However, the presence of the 6s² lone pair and the relativistic splitting of the empty 6p orbitals affects the electronic structures of the Pb(II) center when bound to different ligands. Currently, UV/VIS spectroscopy, X-ray absorption spectroscopy (XAS), and ²⁰⁷Pb NMR spectroscopy are used to give complementary insight into the coordination environment of lead.

XAS is a synchrotron technique for investigating the local structural environments of the metal, geometry, and metal site ligation, while ^{207}Pb NMR spectroscopy is an excellent probe for the Pb(II) coordination environments through the large chemical shift range for this isotope based on different ligand environments of the Pb(II) centers. Though there are a few other techniques that have been used to determine Pb(II) complexes in proteins (e.g., photoelectron, Raman, infrared, and mass spectroscopies), in this chapter we will focus on the application of UV/VIS, XAS, and ^{207}Pb NMR spectroscopies that allow for better understanding of Pb(II)-thiolate interactions.

3.2.1. UV/VIS Spectroscopy of Pb(II) in Small Molecule and Protein

Environments—The coordination of Pb(II) by three cysteinate residues causes charge-transfer electronic transitions in the UV region that can be used to directly monitor Pb(II)-peptide complexes [20]. When Pb(II) binds to cysteines, two intense absorption bands are observed in the UV region: a strong peak with an absorption maximum around 240–280 nm and a moderate signal at a longer-wavelength around 300–350 nm. The molar absorptivity and energy of the latter absorption band can provide quantitative information about lead-protein interactions and are assigned to three or four thiolates bound to a Pb(II) center [20]. Competition experiments with other metal ions result in changes to the ligand-to-metal charge transfer (LMCT) bands when Pb(II) is replaced in the target site. Unfortunately, in most cases, direct determination of Pb(II) binding constants by direct titration is impossible because of the high affinity of this metal for thiolate ligation. Given the extinction coefficient at ~340 nm of $3,500\text{--}4,000\text{ M}^{-1}\text{ cm}^{-1}$, one can only directly determine dissociation constants on the order of $\sim 20\text{ }\mu\text{M}$; however, it is likely that Pb(II) binds to tris cysteinate sites with far greater affinities. Even fluorescence spectroscopy, which can monitor direct complexation of metals at nanomolar levels, has been inadequate to give reliable values. Despite these limitations, the ~340 nm absorption can be used to give relative affinities for Pb(II) as compared to other metals. In most cases, Pb(II) binds much more tightly. Examples of this phenomenon are affinities *versus* Co(II), Zn(II), and Fe(II) [150].

Changes to the binding site also affect the LMCT bands. In a CadC mutant in which one of the cysteines in the metal-binding site has been replaced with a glutamate (C50G), the band is ~15 nm blue shifted compared to the lead-bound wild-type protein [92]. This difference allows for the distinction of Pb(II) S_3 from Pb(II) S_2O sites. A similar trend is observed in the UV/VIS spectroscopy of small molecule Pb(II) complexes containing inorganic thiolate ligands [22, 151, 152]. The spectroscopic fingerprints for Pb(II) bound to a series of small biomolecules (L-Cys, L-Pen, NAC and GSH) in alkaline aqueous solutions established that the environment of the metal center depends on ligand-to-metal ratios, concentrations, and the pH of the solution [22, 151, 152].

For the Cys ligands, the UV/vis spectra displayed absorption maxima at 298–300 nm for the dithiolate Pb(II) $\text{S}_2\text{N}(\text{N/O})$ species at low Cys-to-Pb(II) ratios, while excess ligand led to the formation of tris thiolate Pb(II) S_3N and Pb(II) S_3 (minor) complexes. These were red-shifted and appeared at ~330 nm. The Cys ligand is able to form a five-membered S,N-chelate ring; therefore, in order to shift the equilibrium from the Pb(II) S_2N^- (N/O) to tris thiolate there must be a large excess of free ligand in solution [151]. The blocked amine group in GSH and

N-acetylcysteine (NAC) make the Pb(II) coordination modes at low ligand/Pb(II) mole ratio different than Cys ligand, in which the formation of dimeric or oligomeric $\text{Pb(II)}_2\text{S}_2\text{S}'$ was proposed. However, the corresponding bands are blue-shifted to the tris thiolate Pb(II) environment when a large excess of ligand was added into the solution ($[\text{Pb(II)}(\text{S-NAC})_3]^{4-}$: $\lambda_{\text{max}} = 335 \text{ nm}$, $\text{Pb(II)}(\text{S-GSH})_3$: $\lambda_{\text{max}} = 335 \text{ nm}$) [22]. These studies showed that the LMCT bands of tris thiolate Pb(II) complexes give the most red-shifted spectra compared to the Pb(II)-sulfur centers that have lower numbers of coordinating thiol ligands. This characteristic is one of the spectroscopic features that has been used to probe the tris thiolate Pb(II) center, which is the most common coordination environment of Pb(II) found in proteins.

Glutathione (GSH) also preferentially binds to Pb(II) in a $\text{Pb(II)}\text{S}_3$ coordination geometry between pH 7.5–9.5 [22, 30–33]. However, the LMCT band at pH 7.5 shows the weakest intensity, suggesting an incomplete deprotonation of Cys residues upon Pb(II) binding [33]. When the pH is raised to 8.5 and higher, the thiols are deprotonated and the band becomes more pronounced with an observed λ_{max} of 334 nm ($\epsilon_{334} = 3500 \pm 100 \text{ M}^{-1} \text{ cm}^{-1}$). These parameters are close to the values reported for $\text{Pb(II)}\text{S}_3$ binding into zinc finger proteins and *de novo* 3SCC environments [20, 138].

The binding constants for the zinc finger proteins described in Section 2.4 were determined using UV/VIS spectroscopy. The series of proteins included the cysteine-rich zinc finger consensus domains: CP-CCHH (naturally occurring Cys_2His_2 site), CP-CCHC (consensus $\text{Cys}_2\text{HisCys}$ binding domain), CP-CCCC (consensus Cys_4 site), CP-CCHC, and HIV-CCHC (consensus and HIV nucleocapsid protein containing $\text{Cys}_2\text{HisCys}$ sites) [18, 20]. Only the binding sites that have three or four Cys ligands showed intense bands around 255 nm ($\epsilon_{255} \cong 16,000 \text{ M}^{-1} \text{ cm}^{-1}$) and a well-resolved peak near 330 nm ($\epsilon \cong 4,000 \text{ M}^{-1} \text{ cm}^{-1}$) suggesting $\text{Pb(II)}\text{S}_3$ coordination. When Pb(II) binds to CP-CCHH, the transitions appear blue-shifted [20]. The relative affinities of Zn(II) and Pb(II) for the sites (Figure 6) were determined by titrating Zn(II) into a solution of Pb(II)-zinc finger proteins and following the disappearance of the absorption band at 255 nm.

3.2.2. UV/VIS Spectroscopy of Pb(II) Within *de novo* Designed Peptides—

UV/VIS spectroscopy was used to probe the interactions of Pb(II) with the *de novo* designed 3SCCs described in Section 3.2. The peptides TRIL12C and TRL16C self-assemble to give 3SCCs with Cys residues in the *d* and *a* sites, respectively. A small aliquot of peptide was added into $\text{Pb(II)}(\text{NO}_3)_2$ solutions at pH 8.0 and the $\text{RS}^- \rightarrow \text{Pb(II)}$ LMCT bands were monitored by UV/Vis spectroscopy, confirming the chelation of lead in $\text{Pb(II)}\text{S}_3$ environments [24]. Two intense absorption bands in the UV region were observed: a strong peak with an absorption maximum at 260 nm for TRIL16C and 278 nm for TRIL12C, respectively, and a moderate signal at a longer-wavelength (346 nm for TRIL16C and 343 nm for TRIL12C). The molar absorptivity and energy of the latter absorption band can provide quantitative information about lead-protein affinity and are assigned to $\text{Pb(II)}\text{Cys}_{3/4}$ centers [20].

However, the 3 : 1 ratio of peptides-to-metal ion in the titration curves indicate $\text{Pb(II)}\text{S}_3$ coordination within $\text{Pb(II)}(\text{TRIL12C})_3^-$ and $\text{Pb(II)}(\text{TRL16C})_3^-$ (Figure 12). The lower

energy regions of these spectra are consistent with those observed for Pb(II)-substituted to the metalloregulatory proteins (wild-type *S. aureus* pI258 CadC, Pb(II)-PbrR691, and Pb(II)-AztR), consensus zinc finger peptides (CP-CCHC and CP-CCCC), and the HIV-nucleocapsid protein (HIV-CCHC), implying similar Pb(II) geometries in each of these proteins [20, 23, 87, 153]. However, the higher energy region of the spectra for the Pb(II)-bound 3SCCs and Pb(II)-bound CadC are slightly shifted and show different intensities, indicating that the secondary protein structures around the metal sites may affect the spectroscopy or geometry of the Pb(II)S₃ sites [24].

Soft metal ions, including Pb(II), are believed to bind to the TRI and GRAND peptides under slightly acidic conditions where these peptides have already formed 3SCCs (it should be noted that 2 SCCs are the dominant species below pH 4.5). When Pb(II) is bound at pH 5.5, it is thought to be complexed by one thiolate ligand and two thiol groups, forming, for example, a Pb (II) (TRIL16C) (HTRIL16C)₂⁺ species that contains two coordinated thiol, rather than thiolate, ligands. As the pH is raised, the protons on the two bound thiols are deprotonated simultaneously to form Pb (II) (TRIL16C)₃⁻. The pK_{a2} of these two bound thiol deprotonations can be determined by monitoring the growth of the 342 nm absorption. Figure 13 shows a comparison of the titration curves between Pb (II) (TRIL12C)₃⁻ and Pb (II) (TRIL16C)₃⁻. The curves were best fit with a nonlinear regression model corresponding to a release of two protons (pK_{a2}) from the moiety Pb(II)(H-TRI LXC)₂(TRI LXC)⁺, yielding a Pb (II) (TRILXC)₃⁻. As shown in Table 3, Pb(II) has a lower pK_{a2} of ~12.1 ± 0.2 binding to the *d* peptides (X = 12 or 19) compared to the *a* peptide (pK_{a2} of 12.6 ± 0.2, X = 9 or 16) [24]. These results indicated the protons of Cys residues at a *d* site can be released at a slightly lower pH. If one compares these pK_{a2} values to those determined previously for Cd(II) complexation, also given in Table 3, we see that the thiols are more acidic in the presence of Pb(II). Furthermore, we see the reverse trend in acidities for the *a* and *d* sites, with *a* sites being significantly more acidic (13.6 ± 0.2) than *d* sites (14.6 ± 0.2).

Not only are the pK_a values of the thiols different between TRIL12C *versus* TRIL16C, the affinities for Pb(II) binding are slightly different as well. The binding constant of metal binding to the peptides was analyzed using a nonlinear least square algorithm with the model:

where X is 12 or 16 [24].

Though Pb(II) binds to both peptides with high affinities (> 10⁷ in magnitude), initial determinations of these constants indicated that these values were slightly greater than 10⁷; however, more recent determinations put a lower limit on these values as high as 10¹⁶ [150]. TRIL12C shows slightly tighter binding than TRIL16C, which is a trend that is in contrast with Cd(II) in which the metal binds selectively to the *a* site with 6-fold stronger than the *d* site [24]. It is, however, consistent with the lower pK_{a2} values for *d versus a* sites. The preference of Pb(II) for a *d* site was further confirmed by the Pb(II) titrations into

TRIL9CL19C monitored by ^1H NOESY spectroscopy [154]. The di-substituted TRIL9CL19C has Cys residues incorporated at the ninth position (*a* site) and the nineteenth position (*d* site) simultaneously. Pb(II) was shown to bind preferentially to the L19C site (*d* site) over the L9C (*a* site). Only a 2-fold preference of *d* over *a* was seen for Pb(II) (with Cd(II) > 10-fold preference for *a* versus *d*). The selectivity and affinities of metal binding to each of these sites have later been proposed to depend on the size of the metal pocket, the orientations of Cys and the packing of Leu residues that influences the accommodation of a metal lone pair or a fourth ligand if needed for a 4-coordinate metal center [33, 155].

Crystal structures of apo-CSL9C and apo-CSL19C, representing Cys orientations in an *a* and *d* site respectively, later suggested that the Cys pocket in the *d* site peptide is larger than the *a* site [155]. It could be that the larger atomic radius of the Pb(II) ion (1.33 Å), combined with a lone pair, fits better in a *d* site, while Cd(II) with a smaller atomic radius (0.92 Å) and a fourth ligand would bind to an *a* site. Despite the designed peptides being simpler than the natural proteins, these preliminary observations paved an idea that the relative affinities of different metals in metalloregulatory proteins may be different even though many of these proteins use three Cys ligands as a core feature to bind those metals. Moreover, different locations of the metal sites along a sequence can lead to different selectivities toward metals.

In addition to understanding the affinity and determining structural possibilities for Pb(II) in designed peptides, Zampella et al. subsequently proposed that not only the position along the primary sequence of the Cys pocket mattered for metal affinity, but also the steric considerations of the second coordination sphere for accepting the lone pair could affect the binding constant of complex formation [137]. To test this, a sterically bulky Leu side chain was replaced by Ala at the twelfth position above the metal binding site, yielding TRIL2WL12AL16C, which was thought to remove the steric constraints for metal binding (a Trp was included at the second position as a convenient spectroscopic tag for calibrating peptide concentration). Metal ions that do not have a lone pair (such as Cd(II)) but have a fourth ligand, that occupies the space where the lone pair would be found, should also exhibit this site selectivity.

It was shown that one exogenous water molecule was bound to yield a fully 4-coordinated Cd(II)S₃O species in TRIL12AL16C, whereas TRIL16C, which does not feature a hole above the metal binding site, bound Cd(II) as a mixture of 3- and 4-coordinate species [130, 131]. Parallel studies were done using a crystallographic analogue of the TRI family, called CoilSer peptide. The 3SCC CoilSer derivatives have been shown spectroscopically to bind heavy metals in the interior in analogous manner as TRI family designs [126]. The structural details of the designs will be discussed in detail in Section 3.4. The CSL12AL16C is about 4- to 5-fold higher in stability constant to CSL16C following the trend observed for TRIL2WL12AL16C and TRIL2WL16C [137]. Binding of lead to these peptides may be interpreted as indicating that peptides have a higher affinity for Pb(II) when alanine is in the layer above the *a* site, which was confirmed by ^{207}Pb NMR spectroscopy; however, the quantitative assessment presented in this study may underestimate the binding affinities of Pb(II) in both TRIL2WL12AL16C and TRIL2WL16C. These observations further emphasized that the steric encumbrance at the second coordination sphere of the metal binding site affects the metal affinities and coordination numbers.

DFT calculations were implemented to evaluate the viability of the model that steric hindrance associated with the lone pair of Pb(II) (ACA)₃[−] could lead to site and orientational preferences (ACA is a small modeled peptide with ALAAA-CAALA sequence) [137]. When As(III) was examined, it was suggested that the observed *endo* conformation was adopted to minimize lone pair repulsions. An *endo* configuration is defined when both the β-carbon of Cys and the toxic ion are on the same side of the three atom sulfur plane, while an *exo* configuration refers to a conformation where the β-carbon of Cys is located on the opposite side of the three bound sulfur atoms with respect to the position of the metal. It was shown that without other factors, the preferred Pb(II) conformation was an *exo* isomer as has been seen in enzymes such as ALAD and it was suggested that the higher affinity observed when alanine occupied the layer above the cysteines was a consequence of less steric repulsion for the *exo* Pb(II). The Pb(II) (ACA)₃[−], thus, represented the Pb(II)(CSL9C)₃ system where Cys is occupied at an *a* site (ninth position). Depending on the Cys orientations, two possibilities of *endo* and two possibilities of *exo* might be envisioned in the system of studies (Figure 14).

DFT results reveal the *endo* conformation (*endo a*) that has the Pb(II) ion pointing down toward the C-termini, but Cys rotamers pointing both of their sulfur and β-carbon toward the N-termini is highly preferred [137] (Figure 15). While these calculations gave a reasonable explanation of the data, they were limited in their scope as they only used DFT methods rather than a full bore quantum mechanics/molecular mechanics assessment of the 3SCC. Factors such as hydrogen bonding within the coiled coil or preferred rotamers for cysteines were not fully considered. Thus, the prediction obtained for the 3SCC with alanine above the layer was for conversion to the preferred *exo* conformation (*exo a*) as based solely on the chemical preferences of Pb(II) but as will be described below, the protein environment contribution can generate other structures, especially as the energetic differences between *endo* and *exo* isomers is so small (< 4 kcal/mol) [137]. In fact, the crystallographic description described in the following section will challenge some of the conclusions based solely on the metal sulfur preferences for geometries.

3.2.3. ²⁰⁷Pb NMR Spectroscopy of PbS₃ Centers—Nuclear magnetic resonance (NMR) spectroscopy has become one of the most important methods for evaluating structures of molecules. While many chemists think of this technique primarily for applications using ¹H, ¹³C or ¹⁵N isotopes, it turns out that other elements have NMR sensitive nuclei that may provide outstanding insight into their local structure. We have exploited ¹⁹⁹Hg and ¹¹³Cd nuclei, often in conjunction with another nuclear technique known as perturbed angular correlation of γ-rays spectroscopy (PAC), to probe the binding of these elements to our designed peptides. Using this approach, we were able to distinguish 2- versus 3-coordinate Hg(II) structures and 3- versus 4-coordinate Cd(II) structures that exist at sulfur layers in proteins such as (TRIL12C)₃ or (TRIL16C)₃ [131].

The ²⁰⁷Pb nucleus is also a promising candidate for NMR studies as it has a medium sensitivity (receptivity of the enriched isotope 9×10^{-3} versus ¹H) *I* = 1/2 nucleus with an extremely large chemical shift range (nearly 17,000 ppm) [156, 157]. While ²⁰⁷Pb NMR spectroscopy has been used for some time, it is only recently that this nucleus has been used

to examine homoleptic sulfur environments [41]. This is because for many years signals from a Pb(II)S_3 chromophore had not been detected since the chemical shift range that needed to be explored was so great and the specific resonance so small and narrow. Typical acquisition times, even with enriched samples, can be several hours. However, once the approximate region of the spectrum was established (5361 ppm *versus* PbMe_4 or 2961 ppm downfield of $\text{Pb(NO}_3)_2$) it has now become routine to acquire such spectra [22, 138, 151]. In the below discussion, the chemical shift will be given *versus* PbMe_4 with the $\text{Pb(NO}_3)_2$ value in parentheses.

Jahlilevand and coworkers have examined the complexes of Pb(II) with penicillamine and cysteine using UV/VIS, XAS, and ^{207}Pb NMR to clarify this metal's interaction with these important cellular components [151]. In these studies, they have found that Pb binds with mixed coordination modes including the thiolate sulfur, but also oxygen and nitrogen donors of the carboxylate and amines. These assignments were confirmed based on the relatively high upfield shifts for cysteine and penicillamine ranging from 4794 (1833) to 5252 (2291) ppm. This compares as shown in Figure 16 to 5754 (2793) at pH 8.5 for the PbS_3 values for glutathione [22, 33]. Thus, these small molecules may exhibit a range of different structures, including mixed ligand coordination modes.

A ^{207}Pb NMR spectroscopy study of zinc finger proteins was completed soon after it was realized that NMR could be used to examine such species. The protein fragments HIV-CCHC, HIV-CCGC, and HIV-CCHH (all based on zinc finger domains found in the HIV nucleocapsid protein) were tested [33]. Previously, Godwin et al. had completed a thorough UV/Vis and XAS study that showed that these peptides preferred PbS_3 structures [18]. The ^{207}Pb NMR data for HIV-CCHC exhibited resonances at 5744 to 5790 (2779 to 2829) ppm, values conclusive for the proposed PbS_3 geometry. Unexpectedly, there were two resonances for this protein rather than one as had been suggested by EXAFS and UV/VIS studies. This observation emphasizes the power of ^{207}Pb NMR, as it was able to identify two simultaneously existing conformations (Figure 17) that were undetectable by other probes of lead structure. Thus, while still a relatively slow technique, ^{207}Pb NMR spectroscopy may be useful to establish speciation of systems or longer timescale dynamics.

The first report of ^{207}Pb NMR spectroscopy performed on a Pb(II)S_3 chromophore appeared for designed peptides [138]. In these studies, variants of the cysteine-substituted TRI and CS peptides were interrogated to establish their chemical shift range and the utility of NMR spectroscopy in confirming Pb(II) binding to the peptide. As it turned out, the results were successful beyond our original hopes. Remarkably, these experiments were completed on natural abundance samples, suggesting that natural proteins isolated from cells may be directly probed using this technique. As shown in Figure 18, with designed peptides there was a large chemical shift difference between samples that contained *a* layer cysteines *versus* those that utilized *d* layer substitutions. However, there were only small changes between peptides of different length or composition containing cysteines in the same *a* or *d* layer. Furthermore, while the greatest difference was observed between *a* or *d* layers, there was a useful separation of resonances within derivatives containing cysteine in the same layer and nearby modifications (e.g., $\text{Pb(CSL12AL16C)}_3^-$ at 5555 ppm *versus*

$\text{Pb}(\text{CSL16C})_3^-$ at 5612 ppm). Because of the differences, we were able to assess site-selective binding for Pb(II) to peptides containing two different cysteine layers. In Figure 18 spectrum f, one equivalent of Pb was added to the peptide CSL12AL16CL26C (a peptide containing cysteine in the 16 (*a*) and 26 (*d*) layers and alanine instead of leucine in the layer directly above the *a* site). When one equivalent of Pb(II) was added to this sample, the Pb(II) bound exclusively to the *a* site and when a second equivalent of Pb(II) was added the *d* position became occupied. This experiment elegantly demonstrated that the affinity of Pb(II) was $\text{L12AL16C} > \text{L26C}$.

Previous work, which was confirmed with ^{207}Pb NMR spectroscopy, indicated that Pb(II) had the preference L12C or $\text{L19C} > \text{L9C}$ or L16C , suggesting that the binding order should be an *a* site with an alanine layer above the cysteines is greater than a *d* site which is greater than an *a* standard site (with leucine above) [24, 154]. This prediction was subsequently confirmed, although the intensity difference between $\text{Pb}(\text{TRIL12AL16C})_3^-$ and $\text{Pb}(\text{TRIL16C})_3^-$ was small. Therefore, even though absolute binding constants cannot yet be discerned for these peptides, relative affinities may be deduced from experiments such as these.

A related set of experiments, using both ^{113}Cd and ^{207}Pb NMR spectroscopy, has demonstrated that one can evaluate mixed metal selectivity for these systems [154]. It was shown that Cd(II) binds preferentially to *a* sites and Pb(II) goes to the *d* sites. Thus, one can imagine building designed proteins that can incorporate different metals into different sites selectively and also to measure rates of metal substitution between sites based on metal concentrations or pH.

3.2.4. X-ray Absorption Spectroscopy of Designed Proteins—XAS experiments, especially extended X-ray absorption fine structure (EXAFS) spectroscopy, has been implemented to determine precise metrical parameters for the Pb(II) center. The technique can provide information on ligation type (O,N *versus* S,Cl ligands), coordination number, and metal ligand distances [158]. The Pb(II) L_{III}-edge EXAFS of Pb(II)-bound PbrR691 revealed that the Pb-S bond was 2.67 Å [23], which is in excellent agreement with the Pb(II)–S bond distances determined from in the X-ray diffraction results for [Pb(II)(SPh)₃][−] (2.65 Å), {[Tm^{Ph}]Pb(II)}[ClO₄] (2.69 Å) and other tris thiolate Pb(II) complexes [159–161]. These inorganic complexes illustrated a mononuclear trigonal pyramidal Pb(II) with three thiol-containing ligands arranging in a *hemidirected* fashion. Thus, it suggested that Pb(II) might be bound to native proteins in a similar fashion.

The EXAFS results (~2.64 Å) observed for the designed proteins series that contain either three or four Cys residues (CP-CCCC, CP-CCHC, CP-CCCH, and HIV-CCHC) could be consistent with this viewpoint [20]. The EXAFS spectrum of Pb(II)-bound CP-CCCC is identical to that seen from the tris(2-mercapto-1-phenylimidazolyl)hydroborato Pb(II) complex, [(Tm^{Ph})Pb][ClO₄], suggesting that while four cysteine sulfur atoms are available, the metal binds to only three Cys ligands. The CP-CCCH showed three-coordination; however, lower scattering intensity for peaks at the same distance were observed for Pb(II) bound to CP-CCHC and HIV-CCHC peptides, indicating there could be a mixture of

Pb(II)S₃ and Pb-(OH₂)_x species present in solution or only a homogenous Pb(II)S₂(O/N) species that exists. These results demonstrated that the relative positions of the three Cys ligands in the binding site plays a significant role to stabilize tris thiolate complexes. The CP-CCCH peptide also shows a higher affinity for Pb(II) than those of CP-CCHC and HIV-CCHC, but surprisingly less than the CP-CCCC where all four thiol ligands are present.

Likewise, a single shell of three sulfurs at 2.63 Å was well-fit for Pb(II)-S bond distance of both 3SCC Pb (II) (TRIL12C)₃⁻ and Pb (II) (TRIL16C)₃⁻ peptides [24]. This strongly indicated that both the *d* site (TRIL12C) and *a* site (TRIL16C) Cys environments in 3SCCs bind to Pb(II) in a trigonal pyramidal geometry. This coordination mode is also consistent with Pb(II) bound to CadC in which the Pb(II)-S bond length is 2.66 Å, as reported by Giedroc and coworkers [87, 92]. Apparently, these 3SCC models provide an excellent peptidic environments for a tris thiolate site that could resemble the sites in native metalloregulators.

3.3. Structures of Pb(II) Designed Proteins

Protein X-ray crystallography is a powerful technique that allows one to illustrate the success of designed peptides at the molecular level. Crystal structures not only allow structural insight of the particular metal structure of interest, but could also reveal some hidden features in helical coiled coil scaffolds that may be critical for the metal-protein relationship. In this section, the structural details of a trigonal pyramidal Pb(II) constructed in 3SCC is discussed along with an examination of ligand organization upon metal binding compared to an apo-form. Moreover, the comparison between the designed 3SCC crystal structure with the Pb(II)-bound to the Pb(II)-substituted native yeast ALAD hopes to increase the understanding of Pb(II) binding into Cys-rich sites in different protein environments.

The crystallographic structures of the designed TRI family variants have been obtained using a CoilSer sequence (Table 2). CoilSer was first designed based on TM29 and crystallized by Lovejoy et al., as an antiparallel triple-stranded coiled coil at low pH [162]. By substitution of one or two positions containing hydrophobic residues in the CoilSer peptides with metal-binding ligands (e.g., Cys, Pen or His residue) the Pecoraro group has been able to show that at higher pH the peptides crystallized in a parallel 3SCC manner [127, 143, 145, 147, 155]. For example, the As(III)(CSL9C)₃ (PDB: 2JGO), apo-(CSL9C)₃ (PDB: 3LJM), and apo-(CSL19C)₃ (PDB: 2×6P) are all parallel 3SCCs [127, 155]. The interior metal binding between TRI and CoilSer variants were shown to behave similarly; however, the distinct characteristics that makes CoilSer capable of crystallization are the His residue on the *f* position and Glu residues usually from the *b* and *e* positions of the last heptad to ligate Zn(II) ions on the exterior of the scaffold. This allows for the 3D packing of the 3SCC helices within the lattice. In designs containing a high number of mutations or drastically destabilizing substitutions (e.g., D-amino acids), peptides containing an extra heptad, termed as GRAND-CoilSer series, have been developed to provide more stability to the construct [143, 163].

GRAND-CSL16CL30H was designed as a representative model for Pb(II) bound to an *a* site in a designed 3SCC environment. $\text{Pb(II)}_{\text{S}}\text{Zn(II)}_{\text{N}}(\text{GRAND} - \text{CSL16CL30H})_3^+$ was solved at 1.81 Å resolution in the space group R32. It contains a Pb(II) ion bound as a *hemidirected* trigonal pyramidal Pb(II)S_3 using three Cys ligands at the sixteenth position and a tetrahedral $\text{Zn(II)S}_3\text{O}$ with three His ligands and a water at the thirtieth position (*a* site) close to the C-terminus (Figure 19) [143, 163]. The latter site was originally designed for catalysis, but that will not be emphasized in this chapter.

The bound Cys ligands interact with the Pb(II) center by directing the side chain to the core of the structure and toward the N-termini with an observed χ_1 value of -68.38° (Figure 20). This χ_1 angle is almost identical to the value of -69.20° observed for the major conformation of the unmetallated Cys rotamers in the apo-(CSL16C)₃ (Figure 21), implying that the orientation of Cys in the apo-peptide is highly *pre-organized* for Pb(II) to bind in this trigonal pyramidal geometry. This results in close values of S-γ-Sγ separation between the metallated Pb(II)S_3 structure (3.49 Å) and apo-(CSL16C)₃ (3.29 Å, average).

However, the apo-Cys ligands, in contrast, are *pre-disposed* for trigonal planar Hg(II) because both of the centers require the metal to bind relatively close to the sulfur plane. It would be impossible for Hg(II) to bind to the Cys ligands in the positions they are found in the apo-(CSL16C)₃ without steric clashing. Therefore, a significant rotation of the Cys rotamers to the helical interface is needed to open the metal pocket space for metal sequestration [143]. On the other hand, Pb(II) can accept the apo-structure conformations because the trigonal pyramidal polyhedron allows the metal to be positioned at a distance of 1.69 Å below the metal plane and toward the C-termini of the 3SCC. The fact that both β-carbon planes of the Cys residues and the Pb(II) ion are on the same side of the three atom sulfur plane, this is considered as an *endo* configuration. The best crystallographic model gives the Pb(II)-S bond distance of 2.65 Å, which is within the error of the values determined from X-ray absorption spectroscopy reported for the Pb(Tril16C)_3^- (2.63 Å) [24]. Moreover, the fact that the bond length of the *endo*

$\text{Pb(II)}_{\text{S}}\text{Zn(II)}_{\text{N}}(\text{GRAND} - \text{CSL16CL30H})_3^+$ is consistent with other *exo* structures of Pb(II), such as in ALAD and small molecule Pb(II)S_3 systems, indicates that the Pb(II)-S bond length does not distinguish between the *exo* and *endo* configurations.

Previously, the *endo* conformation was observed for the As(III)S₃ site of the As(III) (CSL9C)₃ crystal structure, while the As(III)S₃ complex had also been predicted to be in an *exo* form in computational studies and inorganic molecules [127]. The small As(III) ion with an atomic radius of As(III) = 0.72 Å (compared to Pb(II) = 1.33 Å) achieves a 2.28 Å As(III)-S bond distance. This shorter bond length does not require the As(III) to be positioned as far below the sulfur plane, with an observed out of plane distance of 1.34 Å. The arrangements of the bound thiols in the first coordination sphere of both structures are shown in Figure 22. In both cases, the ligands are directed toward the core of the structure and upward to the N-termini. The As(III)S₃ has a slightly shorter S-γ-Sγ separation (3.25 Å, average) than the Pb(II)S_3 (3.49 Å) as the size of the bound metal defines the necessary size of the thiol plane. As(III) requires the thiol ligands to orient 10 degrees inward toward the core compared to the ligands in the apo-protein in order to compress the diameter of the

sulfur plane. On the other hand, the binding of the larger Pb(II) causes the thiols to move slightly outward from the core to accommodate a longer M–S bond distance. Regardless of the slight difference in χ_1 dihedral angle values of both structures, the Cys arrangements are almost identical. Both the Pb(II) and As(III) ions are oriented with their lone pairs pointing toward the C-termini of the 3SCC.

These crystallographic observations corroborate well with the reported DFT calculations of the small Pb(ACA)₃ and As(ACA)₃ models, respectively [137]. DFT suggested that the *endo* form is preferable over the *exo* when there are Leu residues above the metal site.

Undoubtedly, the actual crystal structures proved this assumption. Analogously to the small ACA peptide used in the DFT experiments, both of the CSL9C and GRAND-CSL16CL30H derivatives used for X-ray crystallography contain Leu above the Cys site. Touw et al. discovered that the lone pair of As(III) may play an important role in influencing the As(III) coordination sphere within the 3SCC environment [127]. The poorer packing of the 12 Leu residues (one hydrophobic layer below the 9 Cys site) was reasoned to accommodate the lone pair of the *endo*-As(III) rather than the tighter packing of 5 Leu (side chains orienting more toward the helical core). As a consequence, it was thought that As(III) had to orient the lone pair to the C-terminal side of the metal ion so that the lone pair will not suffer from steric clashes with the 5 Leu side chains. The alignment of the trigonal pyramidal As(III)S₃ and Pb(II)S₃ to the apo-structure suggested that metal binding and the effect of the second coordination sphere follow the same trend in both structures (Figure 23).

The fact that Pb(II) and As(III) can adopt the same structure, despite the difference in charge and in size, illustrates the potential importance of *pre-organization* of the Cys ligands prior to metal binding. While the size and lone pairs of the ions may be a contributing factor to metal site location, it is likely that the pre-arrangement of the ligands is the dominant factor for defining the metal position in these systems. This energetic preference potentially comes from the torsion angle restrictions for cysteines in an *a* site of the helical 3SCC system, which does not allow the ligands to orient in a way that can bind Pb(II) in an *exo* conformation nor to be situated on the N-terminal side of the 3SCC.

This torsion angle restriction for cysteines is likely a major factor for defining Pb(II) structures in helical assemblies. In contrast, other secondary or tertiary structures of proteins that are more flexible than the rigid α -helical scaffold may be able to accept the *exo* conformation of tris thiolate Pb(II) complexes more easily. The yeast ALAD X-ray crystal structure, featuring a TIM-barrel by an eight-membered β -sheet surrounded by eight α -helices, has an active site located in a loop connecting β 5 with α 4 at the C-terminal end of the β -barrel and oriented directly to the solvent region [164–166]. The site involves three Cys residues (Cys133, Cys135, and Cys143) which regularly coordinate to Zn(II) with an intervening solvent ligand. However, Pb(II) inactivates the site activity by displacing the tetrahedral Zn(II) with a trigonal pyramidal geometry. The loop region allows Cys to bind Pb(II) in an *exo* configuration where the metal ion is positioned at the opposite side from the β -carbon with respect to the sulfur. The Pb(II) ion appears to be situated above the sulfur plane and is exposed to the solvent region.

The comparison of the *exo* conformation of Pb(II)-ALAD and *endo* conformation of Pb(II)-bound in 3SCC is illustrated in Figure 24. The torsion angles of Cys ligands in ALAD are -142.7° (Cys133), 41.4° (Cys135) and -53.2° (Cys143), which mostly are different from the dihedral angles observed in the $\text{Pb(II)}_s\text{Zn(II)}_N(\text{GRAND} - \text{CSL16CL30H})_3^+$. The variation of the torsion angles in the Pb(II)-bound ALAD system strongly indicates that the loop region provides more flexibility for Cys rotamers to accept a wide range of orientations, rather than the rigidity of the helical 3SCC scaffold. The Pb(II)-S bond distances are 2.7, 2.8, and 2.8 Å for Cys133, Cys135, and Cys143, respectively. The mean S γ -S γ separation between three sulfur atoms is 4.23 ± 0.35 Å (determined from the actual crystal structure using PyMOL software) and the mean S-Pb(II)-S angle is $98.80 \pm 10.86^\circ$ [55]. These observations strongly suggested that the secondary and tertiary level of protein structures serves as a significant role in defining a specific conformation of the trigonal pyramidal geometry of Pb(II) centers in proteins.

Future models for lead interactions must, therefore, consider not only the first coordination sphere ligands, but also the secondary and tertiary structures in which metal binding ligands are located. As an example, Pb(II) bound to the metalloregulatory PbrR691 should take the rotamer restrictions of Cys based on different protein structures into consideration. The PbrR691 belongs to the MerR metalloregulatory protein class. It binds Pb(II) using three Cys residues (Cys78, Cys113, and Cys122) which are also conserved for Hg(II) binding in the MerR protein as determined from the sequence alignment between PbrR691 and MerR (Tn 501) [167]. The recent crystal structure of a Hg(II) bound to MerR (PDB: 4UA1) by Nei-Li Chan and coworkers showed that the trigonal planar Hg(II) site is close to a flexible connecting loop where one of the Hg(II) ligands is located. The second donor is from a helix-to-loop transition and the third Cys is more structurally constrained to the coiled coil dimerization module [168]. This suggests that the flexibility of the loop could possibly affect the Cys rotamers and *endo versus exo* conformations of the Pb(II) complex formed in this protein. On the other hand, the metalloregulatory protein ArsR, a member of the SmtB family which differs from MerR, is helical and must bind As(III) in an irregular C-X-C sequence. Previous models for As(III) binding to ArsR have proposed an *exo*-As(III) configuration [127]. However, the As(III) binding site (Cys32, Cys34, and Cys37) in this protein is in a helical region, so future models for this protein should reconsider the importance of the cysteine rotamers for metal binding. It is quite possible that in the ArsR sequence, the proper rotamers for metal binding cannot be adopted and that this restriction is a contributory factor to the unraveling of the 4-helix bundle upon As(III) association with the protein.

While these crystals structures of designed proteins may inform discussions of the native metalloregulators that have not been structurally characterized in their metallated forms, another important contribution of the studies comes from the perspective of supramolecular chemistry and molecular recognition. This crystallographic study provides structural insight for trigonal pyramidal metalloids that bind into a tris thiolate environment using self associating 3SCCs. As described in the previous section on ^{207}Pb NMR, metal site selectivity along the primary sequence of a protein or spontaneously assembling group of peptides is a major unexplored research area. As we accumulate more structural information

on metalloids in different environments, at different positions along the linear sequence, we will learn the code that helps define molecular recognition at its most basic level. Already, the analysis of metallated and unmetallated *de novo* designed 3SCC peptides allows one to evaluate the extent of ligand *preorganization* prior to metal complexation.

We may now attempt to exploit the clear preference of *a* sites that are *pre-organized* for trigonal pyramidal Pb(II) and As(III) ions. Will this be the basis for Pb(II) having higher affinity for d versus a sites? Conversely, will tetrahedral ions be well accommodated in these different environments? And will these intrinsic apo-protein preferences allow for discrimination of element type, geometry, and coordination number? The answer to these questions should be critical for further understanding the structure-function relationships of metalloids such as Pb(II) and As(III) in biology.

4. GENERAL CONCLUSIONS

In this article we have attempted to integrate our knowledge of the structure and function of known cellular targets for Pb(II) binding to thiolate-rich sites with our fundamental studies aimed at clarifying the spectroscopic and structural behavior of this ion in non-natural constructs that have been prepared based on the first principle ideas. We have seen that using the simple concept of a heptad repeat, assemblies of α -helices (in the form of 3SCCs) serve as excellent vehicles for sequestering Pb(II) into first coordination environments that have either been identified or proposed for most lead-thiolate interactions in proteins and small molecules such as glutathione. Despite the similarity in the first coordination sphere ligand type, distances, and angles, a more comprehensive examination of these systems reveals that there are significant differences between the environments found for Pb in proteins such as ALAD or Zn fingers than coiled coil scaffolds.

First among these differences is that Pb(II) binding in 3SCCs appears to be preorganized with an *endo* conformer being the energetically lowest energy state, despite the apparent preference for *exo* configurations as seen in lead-substituted ALAD or in computational work. In fact, one often sees cysteines that coordinate to metals in β -sheet regions or within loop structures. This is likely because the rigid architecture available with an α -helical bundle requires specific cysteine rotamers to achieve the preferred homoleptic, hemidirected PbS₃-type environment so often found in biology. Furthermore, in metalloregulatory proteins, nature exploits the strong Pb-S bond strength to drive conformational changes to either twist DNA or disrupt helical structures. Such conformational control could not be achieved if a center was constructed within a well designed natural helical bundle. Therefore, we see that while there are apparently many ways that biology could avail itself of binding lead, it is actually the function that drives the type (helix, sheet or loop) of binding site that is found in the protein.

In the case of lead inhibiting existing metalloenzymes the system may be more complicated in that the Pb(II) accepts the structure that was utilized by a different metal. Thus, we see that a tetrahedral Zn(II), as found in ALAD, is replaced with a trigonal pyramidal Pb(II) bound in an *exo* conformation. The lead accepts this structure as the native protein presents an environment that had allowed for a fourth ligand to Zn(II). While it is conceivable that a

tetrahedral ion could accept an *endo* conformation for cysteines, the resultant polyhedron would necessarily be somewhat distorted toward a more trigonal planar system. We conclude that lead is sufficiently promiscuous to complex in this zinc site with little hesitation.

We also see that zinc finger peptides have a strong preference for PbS₃ chromophores as compared to mixed systems that incorporate histidines. However, we also recognize from ²⁰⁷Pb NMR spectroscopy that the types of complexes that form may not be unique, and that two or more conformations could exist. Future work will hopefully assess whether this conformational flexibility could have biological function.

Future work in protein design will need to examine whether the principles derived from the present study for metal binding to α -helices is only narrowly applicable to *a* sites in parallel coiled coils or whether similar chemistry is implicated when lead binds to cysteines in *d* sites or when one has a more natural antiparallel orientation of helices. While the UV/VIS parameters for these divergent centers appear to be similar, the ²⁰⁷Pb NMR shifts are markedly different. Hence, structural studies of this wider range of synthetic constructs will be essential to inform new designs that allow Pb(II) to be understood fully in even these most simple of protein scaffolds.

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ABBREVIATIONS AND DEFINITIONS

A	alanine
ALA	δ -aminolevulinic acid
ALAD	δ -aminolevulinic acid dehydratase
ArsR/SmtB	arsenic resistance regulatory protein family
Baby	peptide containing three heptads
BLL	blood lead level
CaBP	calcium binding protein
<i>cad</i>	cadmium resistance operon
CadA	cadmium resistance efflux ATPase
CadC	cadmium resistance regulatory protein
CaM	calmodulin
CP-CCCC	zinc finger consensus peptide with Cys ₄ site
CP-CCCH	zinc finger consensus peptide with Cys ₃ His site

CP-CCHC	zinc finger consensus peptide with Cys ₂ HisCys site
CP-XCCC	zinc finger consensus peptide with XCys ₃ site
CS	CoilSer peptide containing four heptad repeats
Cys	cysteine
czc	cadmium-zinc-cobalt resistance system
DFT	density functional theory
E	glutamic acid
EPA	United States Environmental Protection Agency
EXAFS	extended X-ray absorption fine structure spectroscopy
GATA	guanine-adenine-thymine-adenine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GRAND	peptide containing five heptads
GRAND-CS	GRAND-CoilSer peptide containing five heptad repeats
GS	glutathione anion
GSH	glutathione
His	histidine
HIV-CCGC	zinc finger HIV domain with Cys ₂ GlyCys site
HIV-CCHC	zinc finger HIV domain with Cys ₂ HisCys site
HIV-CCHH	zinc finger HIV domain with Cys ₂ His ₂ site
HP2	human protamine 2
K	lysine
K_D	dissociation constant
K_i	inhibition constant
L	leucine
LMCT	ligand-to-metal charge transfer
L-Cys	L-cysteine
L-Pen	L-penicillamine

MerR	mercury resistance regulatory protein
NAC	N-acetylcysteine
NMR	nuclear magnetic resonance spectroscopy
NOESY	nuclear Overhauser spectroscopy
PAC	perturbed angular correlation of γ -rays spectroscopy
<i>pbr</i>	lead resistance operon
PbrA	lead resistance efflux ATPase
PbrB/PbrC	lead resistance fusion phosphatase
PbrD	lead resistance lead-binding protein
PbrR	lead resistance regulatory protein
PbrR691	PbrR-homologue
PbrT	lead resistance uptake protein
<i>pbrU</i>	lead resistance inner membrane permease gene
pM	picomolar
ppb	parts-per-billion
Pro	proline
Ser	serine
Sp1	specificity protein 1
TFIIIA	transcription factor IIIA
Thr	threonine
TIM	triosephosphate isomerase
TM-29	synthetic peptide analog of tropomyosin that contains 29 residues with the sequence Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly) ₄ -Lys amide
TRI	peptide containing four heptads
XAS	X-ray absorption spectroscopy
<i>znt</i>	zinc resistance operon
ZntA	zinc resistance efflux ATPase
ZntR	zinc resistance regulatory protein
3SCC	three-stranded coiled coil

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Figure 1. Distribution of Pb(II) in protein crystal structures by ligand (S = sulfur, SC N = side chain nitrogen, MC N = main chain nitrogen). (Adapted from [17]).

Figure 2.
Predominant lead-glutathione complex $[\text{Pb}(\text{II})(\text{GS})_3]^-$.

Figure 3. The active site of ALAD occupied by (a) Zn(II) and (b) Pb(II). (Prepared from PDB 1EB3 (a) and 1QNV (b) [36, 21]).

Figure 4. The CaM-binding site containing (a) Ca(II) and (b) Pb(II). (Prepared from PDB 1EXR (a) and PDB 1N0Y (b) [43, 44]).

Figure 5.

X-ray crystal structures of CaM proteins (a) Ca(II)-CaM (1EXR), (b) Pb(II)-CaM showing only the Pb(II) ions within the EF-binding sites (1N0Y), and (c) Pb(II)-CaM showing all of the coordinated Pb(II) ions including those that are bound opportunistically (1N0Y). (Prepared from PDB 1EXR (a) and PDB 1N0Y (b and c) [43, 44]).

Figure 6. Models of apo-zinc finger consensus proteins. (Generated by modification of PDB PDTB41 using PyMOL [55]). Dissociation constants K_D for Zn(II) and Pb(II) at pH 7.2 [18, 20].

Figure 7. *pbr* resistance in *Cupriavidus metallidurans*. $Pb(II)$ ions are transported into the cell by PbrT. From there they can be coordinated by PbrR, signaling expression of the resistance proteins or coordinated by the metallochaperone PbrD and transported to the efflux ATPase PbrA. PbrA exports $Pb(II)$ into the periplasm when it is precipitated by phosphates produced by PbrB/C. (Adapted from [64, 67]).

Figure 8.
The metal-binding site of ZntR containing two Zn(II) ions in tetrahedral geometries.
(Prepared from the 1.9 Å resolution X-ray crystal structure PDB 1Q08 [81]).

Figure 9. X-ray crystal structure of the CadC dimer. The inducing metal ions are absent. The green sphere represent the structural Zn(II) ions. (Prepared from PDB 1U2W [86]).

Figure 10. Helical wheel diagram for parallel (a) two, (b) three, and (c) four stranded coiled coil peptides. Reprinted with permission from [124]; copyright 2005 American Chemical Society.

Figure 11.

Twisting of the α -helices in a 3SCC along a superhelical axis causing an un-alignment of Leu residues in the *a*, *d*, and *a'* layers in the hydrophobic center. Leu residues are represented as red sticks. Other residues at *b*, *c*, *e*, *f* and *g* are green (oxygen in red and nitrogen in blue). (Prepared from PDB 3LJM [155]).

Figure 12.

Difference titration of Pb(II) into TRI L12C. The change in the absorption at 278 nm is plotted as a function of the equivalents of peptide added in the inset. The experiment was performed at pH 8.0 in the presence of 100 mM tris(hydroxy- methyl)aminomethane buffer while the metal concentration was maintained at 20 μM . (Reproduced with permission from [24]; copyright 2006 Springer, Berlin, Heidelberg).

Figure 13.

pH dependence of complex formation for $\text{Pb}(\text{TRIL16C})_3^-$ (filled squares) and $\text{Pb}(\text{TRIL12C})_3^-$ (open squares). The lines connecting the data points are the fits to the data to extract apparent $\text{p}K_a$ values. Metal concentrations were $10\ \mu\text{M}$ and peptide concentrations were $50\ \mu\text{M}$ as monomer. (Reproduced with permission from [24]; copyright 2006 Springer, Berlin, Heidelberg).

Figure 14.

Scheme showing the four possible orientations of a lone pair-containing metal center within a three-stranded coiled coil. **Top panel:** Two possible orientations for the *endo* configuration in which both the b-carbon of Cys and the toxic ion are on the same side of the three atom sulfur plane. **Bottom panel:** Two possible orientations for the *exo* configuration where the b-carbon of Cys is located on the opposite side of the three bound sulfur atoms with respect to the position of the metal. (Reproduced with permission from [137]; copyright 2012 Wiley-VCH, Weinheim).

Figure 15. DFT-optimized geometries of $[\text{Pb}(\text{II})(\text{ACA})_3]^-$ isomers characterized for *endo* (**left**) and *exo* (**right**) coordination environments. (Reproduced with permission from Ref. ^[137]. Copyright 2012 Wiley-VCH, Weinheim).

Figure 16.

^{207}Pb NMR spectra of Pb(II)-bound reduced glutathione (GSH) in a molar ratio of 1 : 3 (5 mM Pb(II) : 15 mM GSH) at different pHs. All spectra were recorded for 2 h using enriched $^{207}\text{Pb}(\text{NO}_3)_2$ ($^{207}\text{Pb} = 92.4\%$) at 25 °C. (Reprinted with permission from [33]; copyright 2011 Elsevier).

Figure 17.

^{207}Pb NMR spectra of Pb(HIV-CCHC) in a 1 : 1 molar ratio of Pb(II) : peptide at three different pH. The spectra (a) – (c) were recorded at 25 °C and spectrum (d) was recorded at 55 °C. All spectra were recorded for 2 h using enriched $^{207}\text{Pb}(\text{NO}_3)_2$ ($^{207}\text{Pb} = 92.4\%$). (Reprinted with permission from [33]; copyright 2011 Elsevier).

Figure 18.

Natural-abundance ^{207}Pb NMR spectra (104.435 MHz) of Pb(II)-bound three-strand coiled-coil peptides (10–12 mM):

(**a**) $\text{Pb}(\text{BabyL12C})_3^-$, (**b**) $\text{Pb}(\text{CSL12C})_3^-$, (**c**) $\text{Pb}(\text{CSL16C})_3^-$, (**d**) $\text{Pb}(\text{CSL12AL16C})_3^-$, (**e**) $\text{Pb}_2(\text{GrandL12AL16L26C})_3^{2-}$, (**f**) $\text{Pb}(\text{GrandL12AL16L26C})_3^-$. All spectra were recorded for 10–12 hr using natural-abundance $\text{Pb}(\text{NO}_3)_2$ ($^{207}\text{Pb} = 22.6\%$), pH 7.35 ± 0.05 , at 25°C . (Reproduced with permission from [138]; copyright 2010 Wiley-VCH, Weinheim).

Figure 19.

Side view of the trimeric $\text{Pb(II)}_3\text{Zn(II)}_3(\text{GRAND} - \text{CSL16CL30H})_3^+$ structure representing a trigonal pyramidal Pb(II)S_3 site at the 16th position and a tetrahedral $\text{Zn(II)S}_3(\text{H}_2\text{O})$ at the 30th position. Main chain atoms are shown as purple ribbon diagram, 16Cys in the 16th position and His in the 30th position are present as sticks (sulfur = bright yellow, nitrogen = blue). The Pb(II) and Zn(II) ion are shown as a dark and light grey spheres, respectively. The water ligand of the $\text{Zn(II)S}_3(\text{H}_2\text{O})$ site is shown as a small red sphere. (Reproduced from [143]).

Figure 20. PyMOL visualization of the trigonal pyramidal Pb(II)S_3 in the $\text{Pb(II)}_8\text{Zn(II)}_6(\text{Grandin} - \text{CSL16CL30H})_3^+$ structure [55]. **(a)** Top down view from the N-terminus and **(b)** side view of the binding site. Main chain atoms are shown as ribbon diagrams in purple, 12 Leu and 16 Cys side chains in the 16th position are present as sticks (sulfur is yellow). The Pb(II) ion is shown as a grey sphere. (Reproduced from [143]).

Figure 21.

Preorganization of Cys arrangements upon trigonal pyramidal Pb(II) binding in the 3SCC environments. **Top panel:** Representing a top down view from the N-terminus of (a) the apo-protein environment from the apo-(CSL16C)₃ and (b)

Pb(II)_SZn(II)_N(GRAND – CSL16CL30H)₃⁺. **Bottom panel:** An overlay between the apo-(CSL16C)₃ and Pb(II)_SZn(II)_N(GRAND – CSL16CL30H)₃⁺ structures, in (c) a top down view and (d) a side on view. (Reproduced from [143]).

Figure 22.

Comparison of trigonal pyramidal structures of the Pb(II)(S)_3 from

$\text{Pb(II)}_5\text{Zn(II)}_N(\text{GRAND} - \text{CSL16CL30H})_3^+$ and As(III)(S)_3 from the published As(III)(CSL9C)_3 structure (PDB code 2JGO) [127]. Representation of (a) a top down view of the overlay from the N-terminus and (b) a side view of the binding sites. Main chain atoms of $\text{Pb(II)}_5\text{Zn(II)}_N(\text{GRAND} - \text{CSL16CL30H})_3^+$ are colored in purple and As(III)(CSL9C)_3 in red. The sulfur atoms are yellow. The Pb(II) and As(III) ions are shown as grey and red spheres, respectively. (Reproduced from [143]).

Figure 23.

Comparison of the hydrophobic packing below the Cys site of the trigonal pyramidal metal structures compared to the apo-protein. **Top panels:** From top down view of the N-termini, representing the packing of **(a)** 12 Leu below the 9 Cys layer of As(III) (CSL9C)₃, **(b)** 19 Leu below the 16 Cys of apo-(CSL16C)₃ and **(c)** 19 Leu below the 16 Cys of

Pb(II)₅Zn(II)_N(GRAND – CSL16CL30H)₃⁺. **Bottom panels:** Overlays in **(d)** between the As(III)(CSL9C)₃ and apo-(CSL16C)₃, and **(e)** between

Pb(II)₅Zn(II)_N(GRAND – CSL16CL30H)₃⁺ and apo-(CSL16C)₃. Main atoms of

Pb(II)₅Zn(II)_N(GRAND – CSL16CL30H)₃⁺, apo-(CSL16C)₃ and As(III)(CSL9C)₃ are represented as purple, orange and red ribbon diagrams, respectively. Cys side chains are present as sticks in which the thiols are yellow. Leu residues are shown as spheres indicating the packing. Pb(II) and As(III) ions are omitted for clarity. The PDB code for As(III) (CSL9C)₃ is 2JGO [127]. (Reproduced from [143]).

Figure 24.

Crystal structures demonstrating (a) an *endo* configuration of a trigonal pyramid Pb(II)S_3 in the $3\text{SCC Pb(II)}_s\text{Zn(II)}_n(\text{GRAND} - \text{CSL16CL30H})_3^+$ and (b) an *exo* configuration Pb(II)S_3 in the native ALAD. (Prepared (a) from [143] and (b) from PDB 1QNV [21]).

Table 1Zinc finger peptide sequences.^a

Peptide	Sequence			
CP-CCCC	PYKCPECGKSFSQKSDLVKCQRTCTG			
CP-CCCH	PYKCPECGKSFSQKSDLVKCQRT H TG			
CP-CCHC	PYKCPE C GSFSQKSDLVK H QRTCTG			
CP-CCHH	PYKCPECGKSFSQKSDLVK H QRT H TG			
HIV-CCHC	AcHN	VK C FNCGKEG H IARNCRA	CONH ₂	
HIV-CCGC	AcHN	VK C FNCGKEG G IARNCRA	CONH ₂	
HIV-CCHH	AcHN	VK C FNCGKEG H IARN H RA	CONH ₂	

^aBold residues indicate substitutions.

Table 2

TRI family peptide sequences.^{a,b}

Peptide	a	b	c	d	e	f	g	2	9	12	16	19	26	30
BABY	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	G-NH ₂	G-NH ₂
TRI	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	G-NH ₂
TRIL9CL19C	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	G-NH ₂
TRIL12C	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	G-NH ₂
TRIL16C	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	G-NH ₂
TRIL12AL16C	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	G-NH ₂
TRIL19C	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	G-NH ₂
TRIL2WL16C	Ac-G	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	G-NH ₂
TRIL2WL12AL16C	Ac-G	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	WKALEEK	G-NH ₂
GRAND	Ac-G	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK	LKALEEK G -NH ₂
CoilSer (CS)	Ac-E	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	-NH ₂
CSL9C	Ac-E	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	-NH ₂
CSL16C	Ac-E	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	-NH ₂
CSL19C	Ac-E	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	-NH ₂
CSL12AL16C	Ac-E	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	-NH ₂
CSL12AL16CL26C	Ac-E	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	-NH ₂
GRAND-CoilSer	Ac-E	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	LEALEHG -NH ₂
GRAND-CSL16CL30H	Ac-E	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	WEALEKK	HEALEHG -NH ₂

^a Bold residues indicate substitutions.^b C- and N-termini are capped by Ac and NH₂ groups, respectively.

Table 3

p*K*_a values for peptides.

Peptide	Apparent p <i>K</i> _{a2}			
	Pb(II)/TRILXC ^a	Ref.	Cd(II)/TRILXC ^a	Ref.
TRIL9C	12.5	[154]	13.7	[126]
TRIL12C	12.0	[24]	14.6	[24]
TRIL16C	12.6	[24]	13.4	[24]
TRIL19C	12.2	[154]	14.6 ^b	[126]

^aX refers to substitution in the peptide sequence.

^bThis value is for Cd(II)/CSL19C.