Lead Fingers: Pb^{2+} Binding to Structural Zinc-Binding Domains Determined Directly by Monitoring Lead−Thiolate Charge-Transfer Bands

John C. Payne, Marc A. ter Horst, and Hilary Arnold Godwin*

Contribution from the Department of Chemistry, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208-3113

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Abstract: Here, we report that lead−thiolate charge-transfer bands (250−400 nm) can be used to monitor lead binding to cysteine-rich sites in proteins and report the application of this technique to determine the thermodynamics of lead binding to a series of structural zinc-binding domains. These studies reveal that Pb^{2+} binds tightly to structural zinc-binding domains with dissociation constants that range from $K_d^{Pb} = 10^{-9}$ to 10^{-14} M, depending on the number of cysteine residues in the metal-binding site. Competition experiments with Zn^{2+} lead to two striking conclusions: first, the two metals rapidly equilibrate, and second, the ratio of Pb^{2+} to Zn^{2+} bound to a particular site is determined by the relative affinities of the two metals for that site, rather than being under kinetic control. We conclude that Pb^{2+} should be able to compete effectively with Zn^{2+} for Cys_4 sites under physiological conditions. Despite the fact that Pb^{2+} binds tightly to cysteine-rich structural zinc sites, circular dichroism and ^1H NMR studies reveal that Pb^{2+} does not stabilize the correct fold of the peptides.

Introduction

Patients with lead poisoning have *total* blood lead levels (BLLs) that are in the micromolar range.[1] However, the vast majority of the lead (Pb^{2+}) is probably bound by plasma proteins or other cellular components (e.g., glutathione)[2] and the concentration of *free* (or "bioavailable") lead has been estimated to be in the nanomolar to picomolar range.[3] The wide range of symptoms associated with lead poisoning suggests that lead affects multiple targets in vivo. Several proteins have been identified that are activated or inactivated by *femtomolar* to *picomolar* concentrations of Pb^{2+} and hence are likely targets for Pb^{2+} in vivo.[3,4] Because each of these proteins normally binds either calcium or zinc, the hypothesis has been put forth that lead's toxicity arises because lead targets calcium- and zinc-binding sites in proteins.[5−14] However, several central questions concerning the chemistry of lead interactions with proteins need to be addressed before progress can be made toward understanding the toxicity of lead: (1) Out of all of the calcium and zinc proteins found in the body, which ones have a high affinity for lead? (2) Can Pb^{2+} compete effectively with the native metal ions for these binding sites under biological conditions? (3) Is Pb^{2+} binding to proteins under thermodynamic or kinetic control? (4) Does Pb^{2+} binding alter the structure or dynamics of the protein targets?

Previous attempts to address these questions have been hampered by the widespread misconception that Pb^{2+} ([Xe]-$4f^{14}5d^{10}6s^2$) is spectroscopically silent and by the lack of rigorous methodologies that could be used to determine the thermodynamics of lead−protein interactions. Here, we report that when Pb^{2+} is bound to cysteine residues in peptides, the Pb−peptide complex exhibits intense lead−thiolate charge-transfer bands in the region of 250−400 nm. By titrating the peptide with Pb^{2+} and following the appearance of the lead−peptide complex using these charge-transfer bands, the stability of the lead−peptide complex can be determined. In addition, by "back-titrating" the lead−peptide complex with Zn^{2+} and monitoring the disappearance of the lead−peptide complex, the relative affinities of Pb^{2+} and Zn^{2+} for the peptide can be determined. We have used this technique to determine how the affinity of Pb^{2+} depends on the amino acid residues available for metal binding. Lead-binding studies were conducted on a series of three structural zinc-binding domains that differ only in the number of cysteine residues in the metal binding site

* Author to whom correspondence should be addressed.

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(8) By contrast, several workers have suggested that lead's toxicity may arise from the ability of Pb^{2+} to catalyze hydrolytic cleavage of RNA. (See refs 9 and 10.) However, studies on lead-catalyzed cleavage of RNA in vitro indicate that micromolar to millimolar concentrations of free lead are required for efficient cleavage. (See refs 9−14.) Such high concentrations of lead are unlikely to be physiologically relevant.

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(consensus peptides CP-CCHH, CP-CCHC, and CP-CCCC).[15−19] In addition, the lead-binding properties of a zinc-binding domain from HIV nucleocapsid protein (HIV-CCHC) were investigated. This fourth peptide has three cysteines and one histidine in its metal-binding site, but it has a different peptide superstructure than do the consensus peptides.[20−22] The effect of Pb^{2+} binding on the structure of the domains is apparent from one- and two-dimensional NMR studies and circular dichroism studies. These quantitative investigations of lead−peptide complexes challenge several common misconceptions about lead−protein interactions and provide critical insights into the mechanism of lead's toxicity.

Experimental Section

Synthetic peptides were purchased from either Biosynthesis or the Macromolecular Structure Facility at Michigan State University.[17−19,22,23] The molecular weight of each peptide has been confirmed by mass spectrometry.[24] The peptides as received are >70% pure and the remaining purification is conducted in our own laboratory. Immediately prior to use, the peptides are reduced by treatment with 2 equiv of dithiothreitol (DTT) (relative to each equivalent of cysteine present in the peptide) for 2 h at 55 °C. The DTT is then removed and the peptide is purified to homogeneity (indicated by a single peak in the HPLC trace) using reverse phase HPLC. The purified, reduced peptide is then introduced into a chamber that contains an atmosphere of 95% nitrogen and 5% hydrogen and concentrated under vacuum; all subsequent manipulations are conducted under this reducing atmosphere. All buffers are prepared using metal-free reagents and water that has been purified using a MilliQ purification system and subsequently passed through a column containing Chelex media (Sigma) to remove any trace metal contamination. All buffers are purged with helium prior to introduction into the inert atmosphere chamber. Lead and zinc stock solutions are purchased from Aldrich as atomic absorption standards (4.87 mM Pb in 1.0% HNO_3 and 15.5 mM Zn in 1.0% HCl, respectively). A 0.56 M cobalt stock solution is prepared by dissolving $CoCl_2$ (Aldrich, 99.999%) in purified water; the concentration of cobalt is determined using the absorbance at 512 nm ($\epsilon_{512} = 4.8\ M^{-1}\ cm^{-1}$).

Ultraviolet−visible studies were conducted on a Hewlett-Packard 8453 diode array spectrophotometer. Metal-binding studies were conducted in 100 mM bis-Tris, pH 7.0. The concentration of total peptide was determined using the absorbance at 280 or 229 nm (ϵ_{280}(CP-CCHH) = 1400 $M^{-1}\ cm^{-1}$; ϵ_{280}(CP-CCHC) = 1400 $M^{-1}\ cm^{-1}$; ϵ_{280}(CP-CCCC) = 1400 $M^{-1}\ cm^{-1}$; ϵ_{229}(HIV-CCHC) = 3350 $M^{-1}\ cm^{-1}$).[25,26] The concentration of reduced peptide was determined by treating an aliquot of the peptide solution with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB); each free thiol group present in the peptide yields 1 equiv of TNB^{2-} ($\epsilon_{412}(TNB^{2-}) = 14150\ M^{-1}\ cm^{-1}$).[27] Metal stock solutions were diluted in purified, metal-free water and added to the peptide solution in aliquots of 0.1 equiv of metal relative to reduced peptide. In the metal-competition experiments, we ascertained that equilibrium had been reached at each step in the titration by heating at 37 °C until no further change in the spectrum was observed (less than 20 min in each case). The resulting spectra were analyzed using the program SPECFIT,[28] which employs a singular value decomposition algorithm and fits the data using a global least-squares analysis procedure. The buffer (bis-Tris) was selected because it mimics the metal-buffering role of glutathione in cells by forming a stable, soluble complex with Pb^{2+} and thereby prevents formation and precipitation of $Pb(OH)_2$.[2,29] The affinities of the metal ions for the buffer (bis-Tris) are known and were included as parameters in the fit (log β_1 = 1.8 (Co^{2+}), 2.4 (Zn^{2+}), 4.3 (Pb^{2+})).[30] The Pb(bis-Tris) complex only weakly absorbs in the region of 250−400 nm (Figure 1) and hence does not significantly contribute to the spectrum of the Pb−peptide solutions. Nonetheless, the spectrum of Pb(bis-Tris) was included as a known spectrum in the fitting procedure.

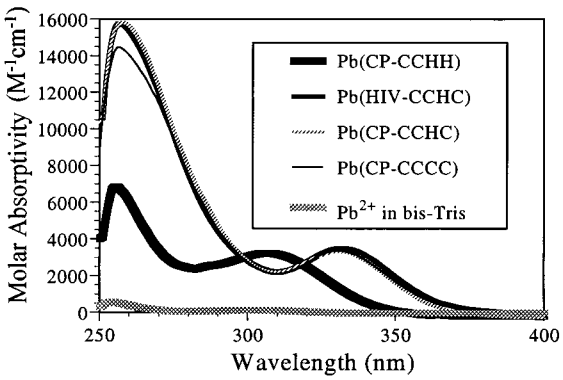

Figure 1. Absorption spectra of the Pb^{2+} complexes of CP-CCHH, CP-CCHC, CP-CCCC, and HIV-CCHC in 100 mM bis-Tris, pH 7.0. The spectrum of the corresponding apo peptide was subtracted from each spectrum. The transitions observed for the lead−peptide species are assigned as lead−thiolate charge-transfer bands in analogy to similar spectra observed for other d_{10}-thiolate complexes.[29,31,32] The intensity of these transitions (ϵ > 1000) is also indicative of charge-transfer bands.

Circular dichroism studies were conducted on a Jasco J-715 spectrophotometer in 50 mM PIPES pH 6.9 in a 0.1 mm cell.

Nuclear magnetic resonance studies were conducted on a Varian Unity Plus 400 MHz NMR spectrometer at 25 °C. Samples were prepared by dissolving lyopholized HIV-CCHC in either 90% H_2O/10% D_2O or 99.9% D_2O and adding Tris-d_{11} to a final pH of 6.7 and a final concentration of Tris-d_{11} of 5−10 mM. Lead and zinc were added to the peptide as stock solutions in D_2O; the pH was readjusted to pH 6.7 using Tris-d_{11} and either NaOD or DNO_3. The total peptide concentrations in the Pb, Zn, and apo samples were 1.3 (+1.4 equiv Pb), 1.2 (+1.4 equiv Zn), and 1.3 mM, respectively.[26] On the basis of DTNB analysis, more than 92% of the peptide in the NMR samples was reduced.[27] One-dimensional spectra were acquired using a 5 mm inverse probe at $T = 25.5 \pm 0.5$ °C. A 1.0−1.5 s low-power pulse was

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(17) The sequence for the zinc finger consensus peptide that has four cysteines in the zinc-binding site (CP-CCCC) is the following: PYKCPECGKSFSQKSDLVKCQRTCTG.

(18) The sequence for the zinc finger consensus peptide that has three cysteines and one histidine in the zinc-binding site (CP-CCHC) is the following: PYKCPECGKSFSQKSDLVKHQRTCTG.

(19) The sequence for the zinc finger consensus peptide that has two cysteines and two histidines in the zinc-binding site (CP-CCHH) is the following: PYKCPECGKSFSQKSDLVKHQRTHTG.

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(22) The sequence for the zinc-binding domain from the HIV-1 nucleocapsid protein (HIV-CCHC) is the following: VKCFNCGKEGHIARNCRA.

(23) Synthetic peptides were purchased from either Biosynthesis (P.O. Box 28, Lewisville, TX 75067-0028) or the Macromolecular Structure Facility at Michigan State University (Department of Biochemistry, East Lansing, MI 48824).

(24) The following molecular weights were obtained by mass spectrometry: CP-CCHH 2962.4 (calculated 2962.4); CP-CCHC 2928.4 (calculated 2928.4); CP-CCCC 2894.3 (calculated 2894.4); HIV-CCHC 2014.6 (calculated 2006.4). Mass spectra were obtained with the peptides from Biosynthesis.

(25) Molar extinction coefficients for CP-CCHH, CP-CCHC, and CP-CCCC were obtained from J. M. Berg and co-workers (personal communication).

(26) The molar extinction coefficient for HIV-CCHC was calculated from the absorbance spectrum of a peptide stock solution that had been quantitated by amino acid analysis. Amino acid analysis was conducted by the Keck Biophysics Facility at Yale University.

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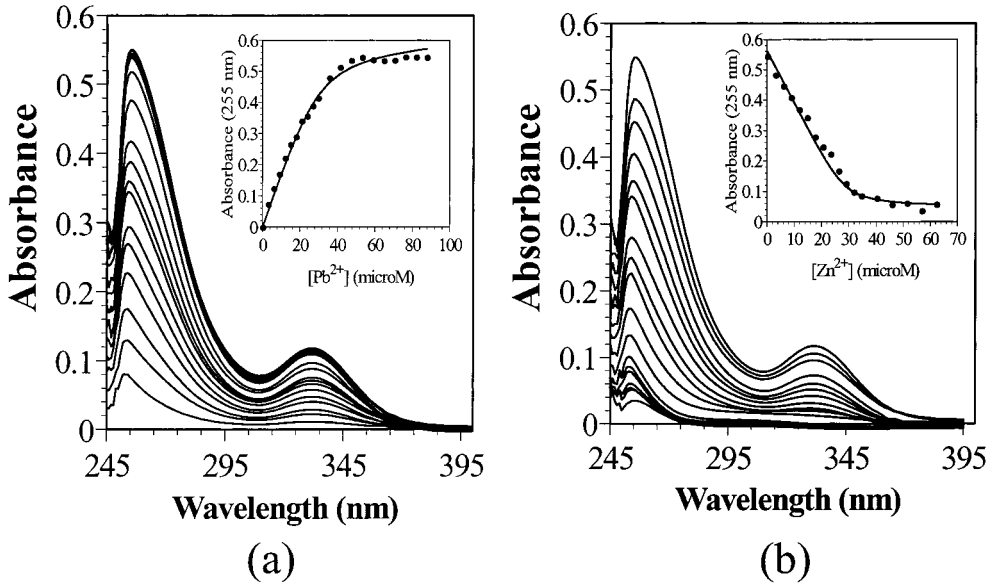

Figure 2. (a) Observed absorption spectra for the Pb^{2+} titration of the CCHC zinc-binding domain from HIV nucleocapsid protein HIV-CCHC and (b) Zn^{2+} titration of the HIV-CCHC zinc-binding domain saturated with Pb^{2+}. The spectrum of the apo peptide was subtracted from each spectrum. Titrations were conducted in 100 mM bis-Tris, pH 6.7. The concentration of reduced peptide at the start of the titration is 29.7 μM.[27] Inset: Fit (curves) to data (circles) yields (a) $K_d^{Pb} < 10^{-9}$ and **(b)** $K_b^{Pb}/K_b^{Zn} = 0.12$.

used for water presaturation. Calibrated 90° pulse widths were used to acquire 128−512 transients of 4K points (acquisition time = 0.41 s) using the first increment of a NOESY pulse sequence (mixing time = 200 ms). The data were zero filled to 16K, Gaussian weighted, and digital filtered upon Fourier transformation. After performing a baseline correction, the peaks were referenced to the water peak at 4.8 ppm. Two-dimensional, double quantum filtered COSY (DQ-COSY) experiments were acquired using a 5 mm inverse probe. A 1.0 s low-power water suppression pulse was applied before the first 90° pulse. A total of 32 transients were acquired at each of the 420 t_1 increments. The 2K × 420 complex data matrix was zero filled to 2K × 2K and processed with a Gaussian function in the direct dimension and a shifted sine bell window function in the indirect dimension.

Results and Discussion

The absorption spectra for the lead−peptide complexes are shown in Figure 1. The large molar extinction coefficients for the bands exhibited by these complexes ($\epsilon_{max} \sim 3000-16000$ M^{-1} cm^{-1}) are consistent with charge-transfer transitions. Similar spectra have been reported previously for other d^{10} metal ions bound to cysteine residues in proteins.[29,31,32] However, Cd−S and Zn−S charge-transfer bands tend to arise at short wavelengths (<250 nm) which are often partially obscured by absorbance of the buffer and hence tend not to be useful for quantifying metal−protein interactions. By contrast, intense Pb−S transitions are observed above 250 nm which can be used to monitor metal binding to relatively dilute samples of peptide (~30 μM). These absorption bands can be used to gain quantitative information about the affinity of Pb^{2+} for the peptides: the spectra obtained upon addition of Pb^{2+} to HIV-CCHC and the resultant titration curve are shown in Figure 2a. In addition, these absorption bands can be used to obtain the relative affinities of Pb^{2+} and Zn^{2+} for a given peptide if Zn^{2+} is titrated into a solution containing the lead−peptide complex (Figure 2b). To test whether this direct methodology provides correct values for the dissociation constant of the lead−peptide and zinc−peptide species, the relative affinities of Pb^{2+} and Zn^{2+} (K_b^{Zn}/K_b^{Pb} and K_b^{Pb}/K_b^{Zn}) obtained for HIV-CCHC as measured from competition experiments between Pb^{2+} and Zn^{2+} were compared to those obtained from competition experiments between each of the metals with Co^{2+} (Table 1).[15,16,33] Similar results were obtained using the two methodologies, but the lead−thiolate charge-transfer bands provide a more sensitive method for probing lead−protein interactions and allow the relative affinities of Pb^{2+} and Zn^{2+} for a given peptide to be determined *directly*.

The lead-binding titrations on the series of isostructural peptides (CP-CCHH, CP-CCHC, CP-CCCC) reveal that the dissociation constant for the Pb−peptide complex decreases markedly as the number of cysteine residues in the metal-binding site increases (Table 1): K_d^{Pb}(CP-CCHH) = 5 × 10^{-11} M; K_d^{Pb}(CP-CCHC) = 8 × 10^{-11} M; K_d^{Pb}(CP-CCCC) = 3.9 × 10^{-14} M. This result demonstrates that hard−soft acid−base

Table 1. Relative Binding Constants Obtained from Competition Experiments: M1P + M2 ⇌ M1 + M2P

peptide	M1	M2	K_b^{M1}/K_b^{M2}
HIV-CCHC	Co	Zn	0.00077 ± 0.00064
	Co	Pb	0.0037 ± 0.0005
	Zn	Pb	12 ± 1
	Pb	Zn	0.12 ± 0.04
CP-CCHH	Pb	Zn	0.1 ± 0.2
CP-CCHC	Pb	Zn	0.04 ± 0.05
CP-CCCC	Zn	Pb	0.046 ± 0.010
	Pb	Zn	43 ± 11

Table 2. Absolute Dissociation Constants (M)

peptide	K_d^{Co}	K_d^{Zn}	K_d^{Pb}
HIV-CCHC	9.0×10^{-8}	7.0×10^{-11}	3.0×10^{-10}
CP-CCHH[a]	6.3×10^{-8}	5.7×10^{-12}	5×10^{-11}
CP-CCHC[a]	6.3×10^{-8}	3.2×10^{-12}	8×10^{-11}
CP-CCCC[a]	3.5×10^{-7}	1.1×10^{-12}	3.9×10^{-14}

[a] Dissociation constants for Co^{2+} and Zn^{2+} obtained from ref 16.

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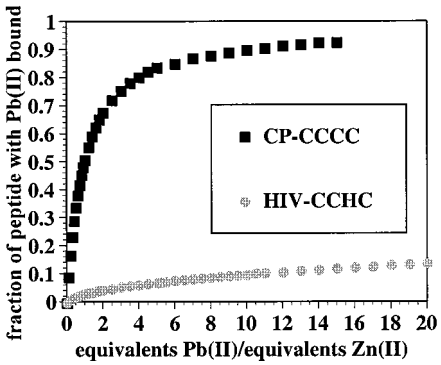

Figure 3. The ability of Pb^{2+} to displace Zn^{2+} from the metal-binding site is determined by the relative affinities for Pb^{2+} and Zn^{2+}. A large excess of Pb^{2+} is not sufficient to displace Zn^{2+} when Zn^{2+} is the thermodynamically preferred metal (HIV-CCHC). By contrast, small numbers of equivalents of Pb^{2+} displace Zn^{2+} almost quantitatively when Pb^{2+} is the thermodynamically preferred metal (CP-CCCC). Experiments were conducted in 100 mM bis-Tris, pH 7.0.

concepts[34] only provide a rough, qualitative prediction for the binding preferences of metal ions to peptides. Although Zn^{2+}, Co^{2+}, and Pb^{2+} are all borderline soft metal ions, Zn^{2+} and Co^{2+} each have roughly the same affinity for the entire series of peptides,[16] whereas Pb^{2+} forms stronger associations with thiols than with imidazoles.[2] These thermodynamic results lead to the expectation that Pb^{2+} should be able to compete effectively with Zn^{2+} for zinc-binding sites that are cysteine-rich. When Pb^{2+} titrations are conducted on peptides that already contain Zn^{2+}, Pb^{2+} displaces Zn^{2+} almost quantitatively if Pb^{2+} is the thermodynamically preferred metal for the peptide (K_b^{Zn}/K_b^{Pb}-(CP-CCCC) = 0.05), but not when Zn^{2+} binds more tightly than Pb^{2+} (K_b^{Zn}/K_b^{Pb}(HIV-CCHC) = 12) (Figure 3). Perhaps the most striking aspect of these studies is that Pb^{2+} and Zn^{2+} rapidly equilibrate on the time scale of these experiments (within minutes at 37 °C). This result is unexpected and of fundamental importance because it provides compelling evidence that lead binding to proteins is under thermodynamic rather than kinetic control. These studies call into question the common assumption in the toxicology literature that Pb^{2+} binds nonspecifically to thiols in proteins and is kinetically inert.

Given that lead can displace zinc from cysteine-rich sites, the question then remains: What is the structure of the Pb−peptide complex? To address this question, Pb(HIV-CCHC) was investigated using one- and two-dimensional nuclear magnetic resonance (NMR) and circular dichroism spectroscopies. Comparison of the one-dimensional ^{1}H NMR spectra of Pb, Zn, and apo(HIV-CCHC) provides several lines of evidence that suggest that the lead−peptide complex is not properly folded (Figure 4). First, the aliphatic resonances of Pb(HIV-CCHC) are not coincident with those of Zn(HIV-CCHC),[20] suggesting that the two complexes do not assume the same conformation. Second, the histidine resonances, which shift significantly when Zn^{2+} is added to apo(HIV-CCHC), do not shift significantly upon addition of Pb^{2+}. Given the precedents in other metalloproteins,[35] this suggests that Pb^{2+} does not bind tightly to the histidine residue. In the native peptide, Zn^{2+} stabilizes the correct fold by locking the histidine residue and the three cysteine residues into a tetrahedral orientation.[20] The lack of a strong Pb−histidine interaction suggests that the amino acids would not be properly

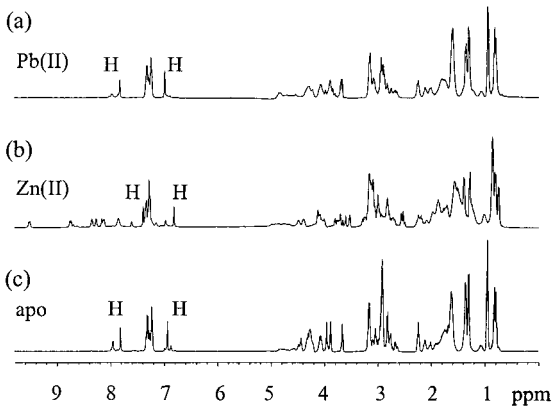

Figure 4. One-dimensional ^{1}H NMR spectra in 90% H_2O, 10% D_2O of (a) Pb(HIV-CCHC) (pH 6.7), (b) Zn(HIV-CCHC)[21](pH 6.7), and (c) apo(HIV-CCHC) (pH 6.7). The total peptide concentration in the Pb, Zn, and apo samples is 1.3 (+1.4 equiv of Pb), 1.2 (+1.4 equiv of Zn), and 1.3 mM, respectively; the samples are buffered with Tris-d_{11}.[26] On the basis of DTNB analysis, more than 92% of the peptide is reduced.[27] Spectra were acquired on a Varian Unity Plus 400 MHz NMR spectrometer using a 5 mm inverse probe at $T = 25.5 \pm 0.5$ °C. A 1.0−1.5 s low-power pulse was used for water presaturation. Calibrated 90° pulse widths were used to acquire 128−512 transients of 4K points (acquisition time = 0.41 s) using the first increment of a NOESY pulse sequence (mixing time = 200 ms). The data were zero filled to 16K, Gaussian weighted, and digital filtered upon Fourier transformation. After performing a baseline correction, the peaks were referenced to the water peak at 4.8 ppm. The resonances corresponding to the aromatic protons on the histidine residue ("H") shift significantly upon Zn^{2+} binding but not upon Pb^{2+} binding, suggesting that, unlike Zn^{2+}, Pb^{2+} does not interact strongly with the histidine. In contrast to Zn(HIV-CCHC), Pb(HIV-CCHC) does not exhibit narrow, well-dispersed amide resonances. This suggests that Pb−peptide does not assume a single, stable conformation.

oriented for folding in the Pb−peptide complex. Third, the amide resonances of the Pb−peptide complex are too broad to be clearly resolved. This suggests that the amide protons rapidly exchange with water on the time scale of the NMR experiment, suggesting that the Pb−peptide complex is fluxional. By contrast, the amide resonances of the Zn−peptide complex are narrow-lined and well dispersed, reflective of the stable, well-ordered structure assumed by the Zn−peptide complex.[20]

The observation that the Pb(HIV-CCHC) complex is not properly folded is also supported by the two-dimensional NMR and circular dichroism spectra. DQ-COSY, NOESY, and TOCSY spectra were acquired at room temperature for the Pb−peptide and Zn−peptide complexes under identical conditions. The Pb−peptide spectrum contains considerably fewer cross-peaks than are observed in the corresponding Zn−peptide spectra (Figure 5) and is indicative of an unfolded peptide. To test whether a single conformation would be induced at lower temperatures, a NOESY spectrum of Pb(HIV-CCHC) was also obtained at 4 °C.[36] The low-temperature data were essentially identical to those obtained at room temperature, suggesting that the Pb−peptide complex remains fluxional even at low temperatures. To test whether the Pb−peptide complex was completely unfolded or whether it was simply interconverting between two or more conformations on the time scale of the NMR experiment, circular dichroism spectra were obtained for Pb, Zn, and apo(HIV-CCHC) (Figure 6). These spectra reveal that on average the Pb−peptide complex is unstructured.

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(36) NOESY spectrum of Pb(HIV-CCHC) at 4 °C (not shown) was obtained at the Biological Sciences Division Nuclear Magnetic Resonance Facility at the University of Chicago.

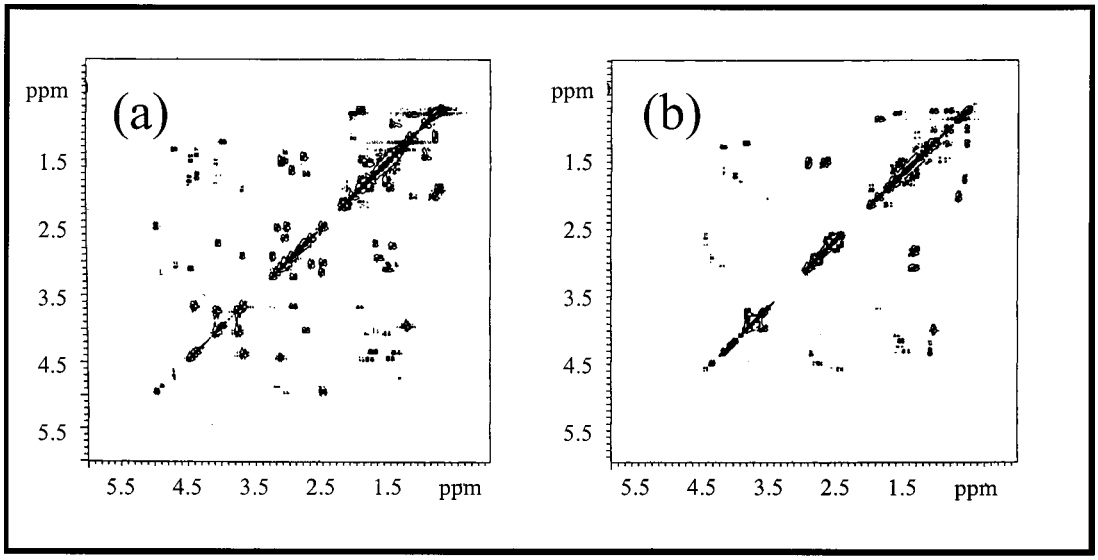

Figure 5. Two-dimensional DQ-COSY ^{1}H NMR spectra in 90% H_2O, 10% D_2O of (a) Zn(HIV-CCHC) (pH 6.7)[21] and (b) Pb(HIV-CCHC) (pH 6.7). The total peptide concentration in the Pb and Zn samples is 1.3 (+1.4 equiv Pb) and 1.2 mM (+1.4 equiv Zn), respectively; the samples are buffered with Tris-d_{11}.[26] On the basis of DTNB analysis, more than 92% of the peptide is reduced.[27] Spectra were acquired on a Varian Unity Plus 400 MHz NMR spectrometer using a 5 mm inverse probe at $T = 25$ °C. In contrast to Zn(HIV-CCHC), Pb(HIV-CCHC) does not exhibit a large number of well-defined cross-peaks, which suggests that Pb−peptide does not assume a single, stable conformation.

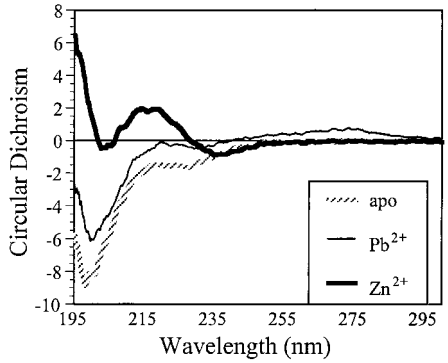

Figure 6. Circular dichroism spectra of apo, Zn, and Pb(HIV-CCHC). Spectra were recorded in 50 mM PIPES, pH 6.9. These spectra reveal that Pb(HIV-CCHC) assumes a random coil conformation, consistent with inactivation of the peptide by Pb^{2+}.

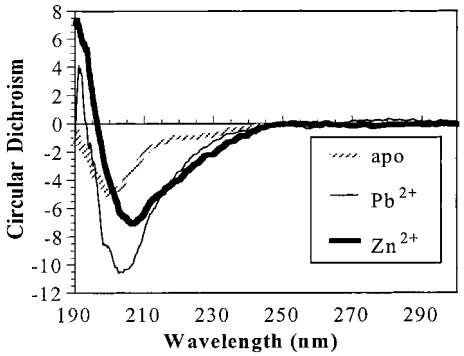

Figure 7. Circular dichroism spectra of apo, Zn, and Pb(CP-CCCC). Spectra were recorded in 50 mM PIPES, pH 6.9. These spectra reveal that the Pb−peptide complex does not assume the same conformation as Zn(CP-CCCC).

Taken together, these studies provide compelling evidence that although Pb^{2+} binds *tightly* to HIV-CCHC, Pb^{2+} does *not* properly fold the peptide. This observation raises two important questions: (1) *How* can Pb^{2+} bind tightly to the peptide if it does not confer proper protein folding and (2) what is the nature of the lead coordination environment when Pb^{2+} is bound to the peptides? The role of Zn^{2+} in a zinc finger domain is purely a structural one: Zn^{2+} stabilizes the correct fold of the domain so that the protein can bind to its target. As a result, Zn^{2+} binding to structural domains results in concomitant protein folding, and the experimentally determined metal ion affinities for these domains necessarily include contributions from both the ΔG of protein folding and the ΔH of the metal−ligand bonds.[16,37] By contrast, Pb^{2+} binding does *not* confer proper protein folding and hence the stability of the Pb−peptide complex must necessarily arise solely from the ΔH of the lead−ligand bonds. The observation that lead forms a stable complex with structural zinc-binding domains is consistent with the prediction that Pb^{2+} has a high affinity for thiol groups in proteins.[2,29] However, the studies reported herein outline the first *direct* spectroscopic measurement of the thermodynamics of these lead−protein interactions.

The observation that Pb^{2+} does not confer the correct conformation to zinc finger domains can be explained because Pb^{2+} and Zn^{2+} exhibit such different coordination chemistries. Pb^{2+} (1.19 Å) is much larger than Zn^{2+} (0.74 Å).[38,39] In addition, Pb^{2+} tends to assume high coordination numbers (six to eight) and is unlikely to bind four ligands in a perfectly tetrahedral orientation.[40] Furthermore, Pb^{2+} does not appear to form a strong bond to the histidine residue in HIV-CCHC, suggesting that the lead does not even coordinate all of the residues that are required for proper folding of this domain. However, even when Pb^{2+} presumably binds all four metal-binding residues (e.g. in CP-CCCC, which contains four cysteine residues), the Pb−peptide complex does not appear to be properly folded: the circular dichroism spectrum of Pb(CP-CCCC) differs significantly from that of Zn(CP-CCCC) (Figure 7). This suggests that the Pb^{2+} coordination environment is probably completed by water or buffer. It is important to note that the measured stability

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constants of the Pb−peptide complexes simply provide the thermodynamics associated with formation of a 1:1 Pb−peptide complex; they *do not* provide any information about the exact speciation or coordination environment of the Pb^{2+} ion within the complex. Future studies will include a detailed investigation of the lead coordination environment (e.g. using resonance Raman and two-dimensional $^{207}Pb-^{1}H$ NMR[41] spectroscopies).

Conclusions

The data presented herein suggest a possible mechanism for the toxicity of lead that differs from the traditional paradigm.[29] On the basis of these results, we propose that Pb^{2+} binds tightly to transcription factors with cysteine-rich zinc-binding sites. We have demonstrated that Pb^{2+} does not constitute a good *structural* substitute for Zn^{2+} and therefore Pb^{2+} is unlikely to stabilize the correct fold of these zinc-binding domains. Because proper folding is required for activity in transcription factors, these data suggest that the ability of Pb^{2+} to displace Zn^{2+} should inactivate this class of proteins. Clearly, studies are needed that test whether Pb^{2+} also displaces Zn^{2+} from sites within *large* proteins (as opposed to isolated domains) and whether metal binding in vivo is also under thermodynamic control.

We propose that when blood lead levels (BLLs) exceed the lead-buffering capacity of cells, Pb^{2+} binds to and inactivates proteins that contain cysteine-rich sites. In particular, members of the steroid receptor superfamily, which contain two tandem Cys_4 zinc-binding sites, are expected to be particularly susceptible to inactivation by Pb^{2+}. This is particularly important in light of a recent analysis of the complete genomes of *S. cerevisiae* and *C. elegans*, which suggests that members of the steroid receptor superfamily play a critical role in growth and development of multicellular organisms.[42] Thus the ability of Pb^{2+} to inactivate proteins that contain Cys_4 structural zinc-binding sites could account for many of the developmental and teratogenic effects of Pb^{2+}.[43−45] In addition, these studies provide an explanation for why Pb^{2+} inactivates the zinc enzyme δ-aminolevulinic acid dehydratase (ALAD) in vivo at very low BLLs, whereas other zinc enzymes are relatively unaffected by Pb^{2+}: ALAD contains an unusual Cys_3 catalytic zinc site which constitutes an unusually good site for Pb^{2+}.[46] Critically, the studies described herein not only challenge the common misconceptions that Pb^{2+} is kinetically inert and spectroscopically silent, but also lay out useful new methodologies for quantitatively studying lead−protein interactions and testing specific hypotheses about the molecular mechanism of lead's toxicity.

Furthermore, the studies presented herein provide interesting insights into lead speciation and the lability of lead complexes under biological conditions. Specifically, the ability of Pb^{2+} and Zn^{2+} to rapidly equilibrate in the presence of structural zinc sites raises the intriguing possibility that the lead speciation is under thermodynamic, rather than kinetic, control. The very high affinity of Pb^{2+} for cysteine-rich sites in proteins begs the question of whether such sites sequester all of the bioavailable Pb^{2+} in vivo and whether the concentration of "free" Pb^{2+} is therefore vanishingly small. We submit the hypothesis that normal cellular components, both small molecules such as glutathione and cysteine-rich sites in proteins, act as a natural "buffering system" for soluble Pb^{2+} in vivo.[2,29] Furthermore, these agents probably act to stabilize the fraction of Pb^{2+} in solution, which would otherwise precipitate as lead hydroxide or lead chloride or be deposited into the bone.[29] In our in vitro studies, the mildly chelating buffer bis-Tris plays this same role. These studies suggest that this pool of loosely coordinated lead is kinetically labile; we expect similar conditions to prevail in vivo for the fraction of lead that is in solution. For instance, the concentration of glutathione in most mammalian cells is >1 mM.[2] This buffering system has an impressive capacity in vivo: the average lead body burden for people in the United States is estimated to be 100 to 1000 times that of prehistoric man[47] and yet the vast majority of these individuals remains asymptomatic. However, relatively small additional increases in BLLs (e.g. from 2 ($\sim$0.1 μM) to 10 μg/dL ($\sim$0.5 μM)) can have a profound effect upon developing individuals.[1,43,48] This suggests that when this buffering capacity is exceeded, Pb^{2+} will start to target critical cellular proteins, even if BLLs only marginally exceed normal levels. Ongoing and future studies in our laboratory are aimed at testing this hypothesis by monitoring the effect of Pb^{2+} on specific transcription factors both in vitro and in vivo.

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