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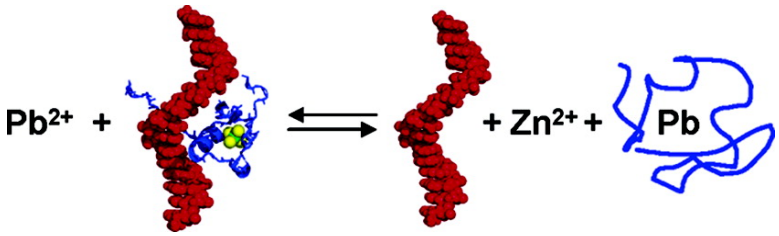
J. Am. Chem. Soc., **2005**, 127 (11), 3751-3759• DOI: 10.1021/ja0464544 • Publication Date (Web): 23 February 2005



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Spectroscopic and Functional Determination of the Interaction of Pb^{2+} with GATA Proteins

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Abstract: GATA proteins are transcription factors that bind GATA DNA elements through Cys_4 structural zinc-binding domains and play critical regulatory roles in neurological and urogenital development and the development of cardiac disease. To evaluate GATA proteins as potential targets for lead, spectroscopically monitored metal-binding titrations were used to measure the affinity of Pb^{2+} for the C-terminal zinc-binding domain from chicken GATA-1 (CF) and the double-finger domain from human GATA-1 (DF). Using this method, Pb^{2+} coordinating to CF and DF was directly observed through the appearance of intense bands in the near-ultraviolet region of the spectrum (250−380 nm). Absorption data collected from these experiments were best fit to a 1:1 Pb^{2+}−CF model and a 2:1 Pb^{2+}−DF model. Competition experiments using Zn^{2+} were used to determine the absolute affinities of Pb^{2+} for these proteins. These studies reveal that Pb^{2+} forms tight complexes with cysteine residues in the zinc-binding sites in GATA proteins, $\beta_1^{Pb} = 6.4\ (\pm 2.0) \times 10^9\ M^{-1}$ for CF and $\beta_2 = 6.3\ (\pm 6.3) \times 10^{19}\ M^{-2}$ for $Pb^{2+}{}_2$−DF, and within an order of magnitude of the affinity of Zn^{2+} for these proteins. Furthermore, Pb^{2+} was able to displace bound Zn^{2+} from CF and DF. Upon addition of Pb^{2+}, GATA shows a decreased ability to bind to DNA and subsequently activate transcription. Therefore, the DNA binding and transcriptional activity of GATA proteins are most likely to be targeted by Pb^{2+} in cells and tissues that sequester Pb^{2+} in vivo, which include the brain and the heart.

Introduction

Lead poisoning is the most common disease of environmental origin in the United States.[1−3] Although lead's extensive use as a paint additive and as an anti-knock agent in gasoline has been discontinued, lead from both past and current industrial uses persists in the environment and remains a significant public health threat.[4] In the United States alone, some 434 000 children exhibit elevated blood lead levels each year due to exposure to Pb^{2+} through oral ingestion of contaminated soil and house dust.[5,6] These children begin to exhibit permanent neurological and behavioral dysfunctions at low blood lead levels (BLLs), with significant neurological damage occurring over the 5−10 μg/dL (0.25−0.5 μM) range.[7,8] In addition, girls exhibit delayed onset of puberty at a BLL of 3 μg/dL.[9] At high BLLs (≥40 μg/dL), adults experience fertility problems (lowered sperm count and abnormal sperm for men; infertility and miscarriage for women), as well as neurological dysfunction and anemia.[3,10,11] A recent analysis using the Second National Health and Nutrition Examination Survey (NHANES II)[12] found that past lead exposure at moderate BLLs of 20−29 μg/dL correlated

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with greatly increased circulatory and cardiovascular mortality (39% increase compared to control group) in adults.[13] To date, no molecular explanation for this effect has been provided. Similarly, no molecular targets have been reported that could account for the observation that hypertension correlates with both moderate BLLs (10−20 μg/dL) and *bone* lead levels.[11,14] An understanding of the molecular mechanism of lead poisoning is needed to develop treatments for those individuals that have already been exposed to lead and to guide health policies in the prevention of lead poisoning.[12,13]

Pb^{2+} mediates its deleterious effects in part by acting at Zn^{2+}-binding sites in vivo and thereby altering the activity of Zn^{2+}-dependent proteins. Pb^{2+} has been shown to inhibit the Zn^{2+}-dependent enzyme 5-aminolevulinate dehydratase (ALAD, the second enzyme in the heme biosynthesis pathway) at femtomolar concentrations in vitro (K_i = 0.07 pM).[15] Correspondingly, ALAD activity in vivo decreases linearly with increasing BLLs.[16] X-ray crystallography confirmed that Pb^{2+} substitutes at the active site of ALAD, which contains an atypical Cys_3 catalytic zinc site.[17] Both in vivo and in vitro evidence indicate that Pb^{2+} can also act directly on proteins that contain structural zinc-binding domains, and that this may contribute to Pb^{2+}-induced alterations of gene expression.[18−23] Zawia and co-workers demonstrated that the timing of DNA binding to the transcription factor Sp1, which contains three Cys_2His_2 zinc fingers, is altered in Pb^{2+}-exposed cultured PC-12 cells and rat pups.[23] This shift in Sp1 DNA binding to an earlier time period was accompanied by altered expression patterns for Sp1 regulated genes. Evidence indicating that the effect of Pb^{2+} on Sp1 is due to its interactions with the structural zinc-binding domain is provided by the fact that neither the DNA-binding activity nor the accompanying gene-regulation activity of transcription factors that lack zinc-dependent structural domains (e.g., NFκB) were affected by Pb^{2+}.[23] Additional support for the conclusion that Pb^{2+} can alter the function of structural zinc-binding domains comes from in vitro experiments demonstrating that Pb^{2+} alters the DNA-binding activity of a synthetic Sp1 peptide, recombinant human Sp1, and TFIIIA.[18,21]

Likewise, studies on a peptide from the HIV nucleocapsid protein, which contains a $Cys_2HisCys$ zinc site (HIV-CCHC), revealed that Pb^{2+} does not induce the Zn^{2+}-stabilized structure required for RNA binding, thus providing an explanation for the disruption of the structural zinc-binding domain's activity by Pb^{2+}.[19] In addition to showing that Pb^{2+} does not stabilize the same structure as Zn^{2+}, Godwin and co-workers measured the affinity of Pb^{2+} for different types of structural zinc-binding domains using zinc finger consensus peptides with Cys_2His_2 (CP), $Cys_2HisCys$ (CP-CCHC), and Cys_4 (CP-CCCC) metal-binding residues.[19,24] These experiments established that while Pb^{2+} binds tightly to Cys_2His_2 sites, Pb^{2+} has the highest affinity for Cys_4 structural zinc-binding sites. In the synthetic peptide system, Pb^{2+} bound to the Cys_4 site more tightly than Zn^{2+}.[19] These results pointed to the need for detailed studies on the interaction between Pb^{2+} and naturally occurring proteins that contain cysteine-rich zinc-binding sites.

One important class of transcription factors that contain cysteine-rich structural zinc-binding domains is the Cys_4 GATA proteins, named eponymously for the GATA DNA base recognition sequence to which it binds. The vertebrate GATA family currently totals six members that perform vital functions in a variety of tissues.[25−36] GATA proteins play significant roles in several tissues affected by lead poisoning, including the hematopoietic, nervous, urogenital, and cardiovascular systems. In the hematopoietic system, GATAs-1, -2, and -3 have been shown to be absolutely required for the development of blood cells.[27,37−43] GATA-2 and GATA-3 play roles in the development of the nervous system.[44−46] Multiple GATA proteins are implicated in the genitourinary systems,[28,29] including development of the female urogenital system[47] and sexual dimorphism.[48−50] GATA-4 and GATA-6 are also implicated in reproductive processes in the ovaries.[51,52] In the heart, GATA-4 regulates many structural and regulatory proteins,[34] and thus

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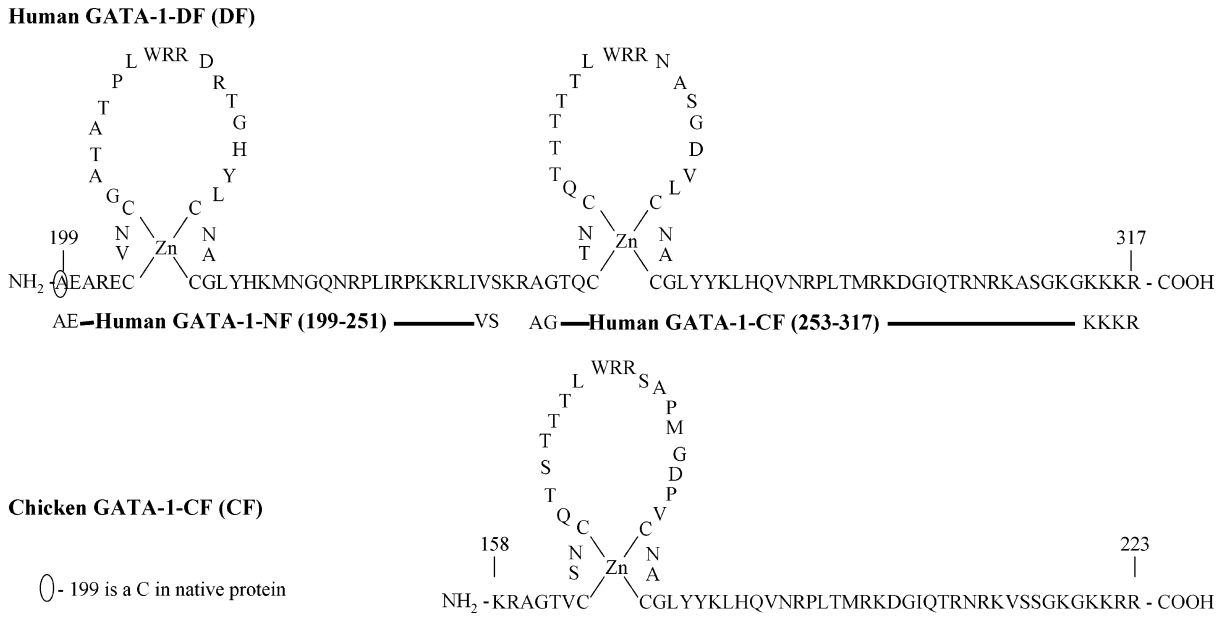

Figure 1. Amino acid sequences of human GATA-1-DF (DF) and chicken GATA-1-CF (CF) are presented as schematics. The human GATA-1-NF (NF) is represented by the labeled solid line underneath its corresponding finger in the DF schematic. The human GATA-1-CF finger is represented by the labeled solid line underneath its corresponding finger in the DF scheme. The CF fingers from the chicken and human species are 86.4% identical.[99] Reprinted from ref 76.

plays critical roles in healthy cardiac development.[36] In addition to regulating development of a healthy heart, GATA-4 plays vital roles in the development of cardiac hypertrophy, the development of a diseased heart, and stress response in the heart.[53−63] Interference with these activities by lead could account for many of the symptoms associated with lead poisoning and would explain recent evidence that moderate Pb^{2+} exposure is associated with a greatly increased rate of cardiovascular disease.[13]

Each of the six vertebrate GATA proteins features two tandem GATA domains, termed the N-terminal GATA finger (NF domain) and the C-terminal GATA finger (CF domain) and collectively known as the double GATA finger (DF), that bind to DNA (Figure 1).[25,26,31,64−69] Each of the two GATA fingers has a Cys-X_2−Cys-X_{17}−Cys-X_2−Cys amino acid sequence. Zn^{2+} coordinates in a tetrahedral geometry to the four cysteines and induces the domain to fold into the GATA structure of an N-terminal base of irregular β sheets followed by an α helix and an extended loop.[70,71] NMR studies of the C-terminal domain from chicken GATA-1 revealed that zinc's ability to bind in a tetrahedral fashion is essential to GATA secondary structure.[70] This structure shows that the first two cysteines are located in the N-terminal base of the domain, and the second two cysteines are in the α helix such that when Zn^{2+} binds to these residues in tetrahedral geometry, it controls the orientation of the α helix with respect to the β sheets.[70] CD spectroscopy revealed that Co^{2+} can replicate the Zn^{2+}−stabilized structure of the N-terminal domain of murine GATA-1, and DNA-binding studies reveal that Co^{2+} can also replicate the DNA-binding activity of GATA-1-CF.[72,73] Fe^{2+} and Cd^{2+} both stabilize the binding of GATA-1-CF to DNA, while Mn^{2+} does not.[73]

No studies have been reported to date between GATA and the toxic metal lead (Pb^{2+}). Here, we report the affinity of Pb^{2+} for the C-terminal domain of chicken GATA-1 and the double-finger domain of human GATA-1 (henceforth referred to as CF and DF; see Figures 1 and 2) measured through spectroscopically monitored metal-binding and metal-competition experiments. CF and DF were chosen for this study because there exists a body of structural, DNA-binding, and metal-binding data that will facilitate determination of the relevance of GATA proteins to lead poisoning. For CF, the structure was previously

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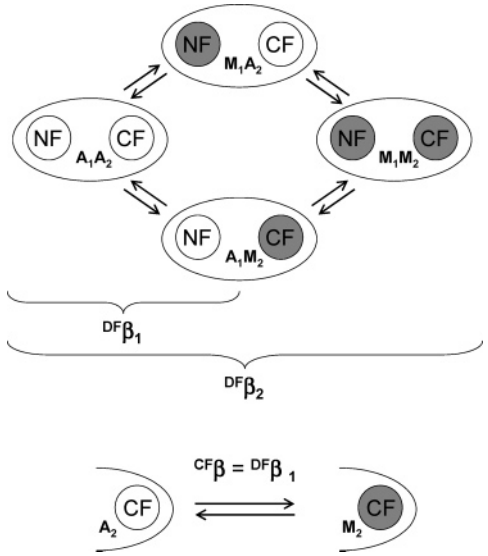

Figure 2. Scheme depicts the thermodynamic possibilities for two metal ions binding to a protein with two metal-binding sites with stoichiometric stability constants (β_1 and β_2) identified. "A" refers to apo (metal-free) sites, and "M" refers to metal bound to the site. A similar diagram pertains to the situation where one metal is replacing a second metal, but A would be replaced with M′. The subscripts for the site occupancy markers (A_1, A_2, M_1, M_2) refer to the site number; the subscripts in the stoichiometric stability constants (β_1 and β_2) refer to the metal stoichiometry. The following definitions are used:[100] β = overall stability constant = $([ML_x^{n+}]/[M^{n+}][L]^x)$; $\beta_1 = ([ML^{n+}]/[M^{n+}][L])$; $\beta_2 = ([ML_2{}^{n+}]/[M^{n+}][L]^2)$; etc.

determined by NMR,[70] while for CF and DF, the DNA-binding constants have been measured for a number of DNA recognition elements.[73−75] Metal-binding constants have also been reported for the interactions of Co^{2+} and Zn^{2+} with these constructs.[76] The DNA-binding domains of GATA family members exhibit high sequence similarity and functional experiments have shown that the activities of the individual GATA-4 domains are similar to those of GATA-1.[31,33,66,77] As a result, the Pb^{2+}-binding affinities for CF and DF reported herein can be used as the basis for understanding the susceptibility of GATA proteins to disruption by Pb^{2+}. We have determined that Pb^{2+} binds to these domains with high affinity and within an order of magnitude of the affinity of Zn^{2+} for these constructs. Moreover, Pb^{2+} is able to displace Zn^{2+} from CF and DF under physiologically relevant conditions.

The biological significance of the ability of Pb^{2+} to bind to GATA would likely be brought about by modulation of the GATA/DNA interaction in the presence of Pb^{2+}, as the metal-binding site anchors the folding of the DNA-binding domain. We have found that, in addition to the ability of Pb^{2+} to bind to apo-GATA nearly as tightly as Zn^{2+}, Pb^{2+} is also able to diminish GATA function. We have observed a decrease in DNA binding by GATA when Pb^{2+} is titrated into an in vitro gel mobility shift assay for GATA-binding activity and an inhibition of GATA-mediated transcription activation in a model yeast system when Pb^{2+} is added to growing cell cultures.

Materials And Methods

GATA Proteins. The sequences of recombinant chicken GATA-1-CF protein (residues 158−223, CF) and recombinant human GATA-1-DF protein (residues 199−317, DF) are provided in Figure 1.[76] CF and DF were expressed and purified as previously described.[70,74] After purification, the recombinant proteins are in their reduced apo-forms and are >95% pure (single HPLC peak characterized by amino acid analysis and N-terminal sequencing).

General Methods and Reagents Used in Metal-Binding Titrations. To determine accurately the concentrations of CF and DF proteins prior to binding Zn^{2+} or Pb^{2+}, absorbance-monitored metal-binding titrations and Co^{2+} concentration analyses were performed as described earlier[76] under an inert atmosphere in Teflon-stoppered quartz cuvettes and recorded using a Cary 500 UV−vis−NIR spectrometer. All nonmetal solutions used in the titrations were prepared with metal-free reagents and water (milliQ water passed over Sigma chelex resin) and were purged with Ar prior to being transferred into a Coy inert atmosphere chamber (glovebox, 95% N_2, 5% H_2). The following stock metal solutions were stored in the glovebox and diluted for use as titrants: Pb^{2+} atomic absorption standard (Aldrich, 4.85 mM Pb^{2+} in 1% HNO_3); Zn^{2+} atomic absorption standard (Aldrich, 15.25 mM Zn^{2+} in 1% HCl). All titrations were performed under an inert atmosphere in 100 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (bis-Tris), pH 7.0. Reduced apo-GATA (CF or DF) was dissolved in chelexed water immediately prior to titration without further reduction and/or purification. The concentration of the GATA proteins used in the titrations was calculated based on the absorption at 740 nm of an aliquot of GATA solution in the presence of excess Co^{2+} (calculated $\epsilon_{742\ nm} = 944\ M^{-1}\ cm^{-1}$ for Co^{2+}−CF, calculated $\epsilon_{740\ nm} = 2408\ M^{-1}\ cm^{-1}$ for $Co^{2+}{}_2$−DF).[76]

Direct Pb^{2+} Titrations and Pb^{2+}/Zn^{2+} Competition Titrations. Reduced, apo-GATA (∼10−20 μM) was placed in a sealed cuvette, and Pb^{2+} atomic absorption standard was added (0.6−1 mM, 0.1 molar aliquots per CF and 0.2 molar aliquots per DF). An absorption spectrum was collected after each addition of Pb^{2+} until 2.5 molar equiv of Pb^{2+} was added for CF or 5.0 molar equiv Pb^{2+} was added for DF. A final data point was obtained at saturation (8 molar equiv of Pb^{2+} per GATA metal-binding site). Zn^{2+} atomic absorption standard was then added to the cuvette in 0.1 molar aliquots per CF or in 0.2 molar aliquots per DF. To ensure that equilibrium had been reached, the cuvette was incubated for 10 min at 37 °C after each addition (until the absorption spectrum ceased to change). After addition of lead, aliquots of Zn^{2+} were added for purposes of competition until approximately 5 molar equiv of Zn^{2+} was added per CF or 10 molar equiv was added per DF, and the intensity of the charge-transfer bands was monitored by UV−vis spectroscopy after each addition.

Zn^{2+}/Pb^{2+} Competition Titrations. Reduced, apo-GATA protein (∼10−20 μM) was placed in a sealed cuvette, and Zn^{2+} atomic absorption standard (0.01525 M) was added to the apo-GATA solution in approximately 1.1 molar ratio per CF or in approximately 2.2 molar ratio per DF. Initial additions of Pb^{2+} titrant were in 0.1 molar aliquots per CF or in 0.2 molar aliquots per DF, and Pb^{2+} additions continued until 20−40 molar equiv of Pb^{2+} was added per CF or DF or until the solution pH dropped below 6.5. An absorption spectrum was collected after each addition of Pb^{2+}. To ensure that equilibrium had been reached with each addition, the cuvette was incubated at 37 °C until the absorption spectrum ceased to change (approximately 10 min).

Analysis of Absorption Data using Specfit/32. The metal-binding data that were obtained from the titrations were analyzed using the program Specfit/32.[78] Specfit/32 calculates binding constants using a binding model analyzed by factor analysis method in which all colored species are included in the model. The affinities of M^{2+} for bisTris

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(78) SPECFIT is a product of Spectrum Software Associates and is owned solely by the authors, Robert Binstead and Andreas Zuberbuhler.

(Pb^{2+} log $\beta_1 = 4.3$; Zn^{2+} log $\beta_1 = 2.4$) were also included as known values in the model.[79]

Gel Mobility Shift Assay. CF (25 nm) in a 1:1 complex with Zn^{2+} was incubated with 25 nm of 5′-^{32}P-labeled DNA (Integrated DNA Technologies, Inc., IA) in 50 mm Tris pH 7.0, 6 μm sodium phosphate pH 7.0, 0.125% NP-40, 2 mm EDTA, 20 μg/mL poly(dI-dC), and 10% glycerol. The DNA was 5′-end-labeled with γ-(^{32}P)ATP using T4 polynucleotide kinase (New England Biolabs, MA) according to the manufacturer's instructions. The sequence of the DNA used was 5′-AGCTTCGGTTGCAGATAAACATTGAATTCA-3′. Pb^{2+} was added in increasing concentrations (0−2.5 μm) and incubated with Zn^{2+}−CF at 25 °C for ∼5 min prior to addition of the DNA; the entire mixture (Pb^{2+}, Zn^{2+}−CF, plus DNA) was incubated at 25 °C for a total of ∼30 min.

The CF/DNA complexes were analyzed by 8% native gel electrophoresis in 10 mM Tris/10 mM HEPES pH 7.5 and 1 mM EDTA. The gel electrophoresis was conducted in 10 mM Tris/10 mM HEPES pH 7.5 and 1 mM EDTA at 150 V, 4 °C. The 5′-^{32}P-labeled DNA was detected using a Storm PhosphorImager (Amersham Biosciences, NJ). The density of the bands was determined with the ImageQuant program (Molecular Dynamics). The percent free DNA was calculated as $D_f/(D_b + D_f)$, where D_f is the measured density of the free DNA and D_b is the measured density of the bound DNA.

In vivo Binding of GATA to DNA in the Presence of Pb^{2+}. A "one-hybrid" assay for binding of the CF to DNA was constructed using a commercial system (Clonetech "Matchmaker") by integrating four repeats of a GATA-binding site into the promoter of a LacZ reporter gene on a yeast integrating plasmid (pLacZi). This construct was integrated into *Saccharomyces cerevisiae* according to the supplier's protocol. The CF cDNA was fused in-frame with the yeast Gal4 activation domain in the expression plasmid pGAD424. When this expression plasmid is introduced into yeast carrying the GATA-LacZ reporter gene, it activates expression of LacZ as assayed by standard β-galactosidase assays[80] in a GATA site-specific manner. To test the effect of Pb^{2+} on GATA-mediated LacZ expression, yeast bearing the GATA-LacZ reporter and the CF/GAL4 activation domain vector were grown to saturation in small overnight cultures. One milliliter of this starter culture was added to 50 mL of minimal yeast growth media containing increasing amounts of Pb^{2+} and grown to prelog phase culture conditions (8 h to overnight) until the culture density gave an absorbance at 600 nm of 0.8−1.0. At this point, cells were harvested and assayed for β-galactosidase activity by monitoring hydrolysis of the colorigenic substrate *o*-nitrophenylgalactoside (ONPG). Activities reported are absorbance at 420 nm per minute of assay time, normalized to the initial cell culture density. For comparison, yeast bearing the expression pGAD53m, a control plasmid which expresses the GAL4 activation domain fused to the mouse p53 protein, along with a p53/LacZ reporter (control plasmids supplied by Clonetech), were also grown and treated in the same manner by addition of Pb^{2+} and assayed for β-galactosidase activity.

Results

Direct Pb^{2+} Titrations. Pb^{2+} coordination to CF and DF can be monitored directly through intense ultraviolet bands that emerge as Pb^{2+} coordinates to cysteines in the Zn^{2+}-binding site, which arise from a combination of charge-transfer transitions (between mixed s and p orbitals with both metal and ligand character) and Pb^{2+} intraatomic transitions (Pb^{2+} 6s orbital to Pb^{2+} 6p orbital).[81] As Pb^{2+} is titrated into either CF or DF, these intense bands appear at 264 and 335 nm for CF and at 263 and 336 nm for DF. Representative data from a Pb^{2+} titration of DF are shown in Figure 3A. The spectra of CF and DF saturated with Pb^{2+} are shown in Figure 3B. These direct Pb^{2+} titrations were used to determine the stoichiometric ratio of Pb^{2+} complexes with CF and DF. Pb^{2+} saturates CF at 1 molar equivalent. Lead binds too tightly to the cysteines in these sites to obtain a binding constant from the direct titration of Pb^{2+}; only a lower limit for the binding constant can be obtained from this experiment. Using a 1:1 model to fit the absorption data from Pb^{2+} titrations of CF yielded the lower limit for Pb^{2+}−CF affinity of ${}^{CF}\beta^{Pb} \geq 2.5 \times 10^9\ M^{-1}$. A comparison of the fit to data for a representative Pb^{2+} titration of CF is shown in Figure 3C. Pb^{2+} saturates DF between 2.0 and 2.4 molar equiv. To determine whether Pb^{2+} is binding to DF in a 2:1 stoichio-

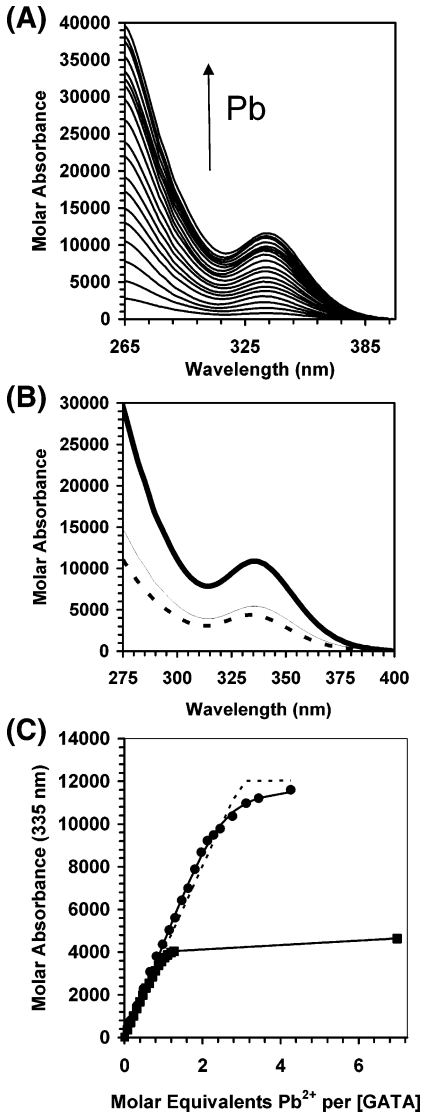

Figure 3. (A) Absorption spectra collected during a Pb^{2+} titration of DF (11 μM) in 94 mM bisTris, pH 7.0, show lead−thiolate bands that increase in intensity as Pb^{2+} coordinates to cysteines in the DF metal-binding sites. Similar data for CF are provided in the Supporting Information. (B) Molar absorptivity spectra of $Pb^{2+}{}_2$−DF (thick dark line), half of $Pb^{2+}{}_2$−DF (thin gray line), and Pb^{2+}−CF (dashed line) are shown. (C) Comparison of calculated fits (lines) to observed data (circles/squares) at 335 nm for Pb^{2+} titrations of DF (11 μM) (●) and CF (18.7 μM) (■). For the DF titration, the fit obtained using the 2:1 Pb^{2+}/DF model (solid line; ${}^{DF}\beta_2^{Pb} \geq 5.0 \times 10^{17}\ M^{-2}$) is quantitatively and qualitatively superior to that obtained using a 3:1 Pb^{2+}/DF model (dashed line, ${}^{DF}\beta_3^{Pb} \geq 9.2 \times 10^{27}\ M^{-3}$).

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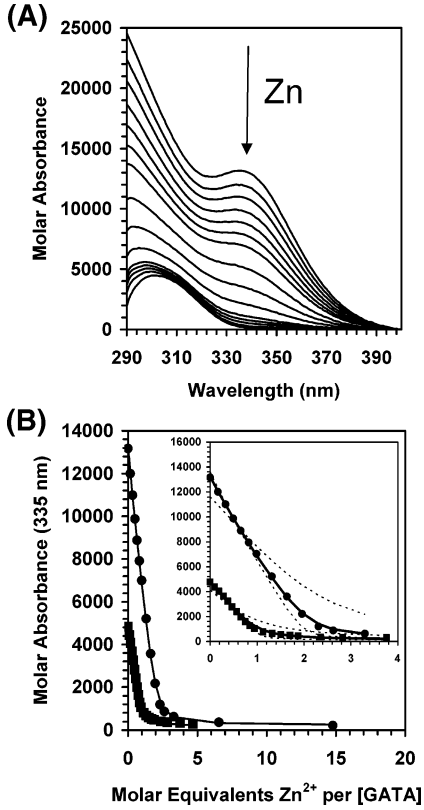

Figure 4. (A) Absorption spectra collected during a Zn^{2+} titration of DF (11.0 μM) in 94 mM bisTris, pH 7.0 at 37 °C and the presence of approximately 16 molar equiv of Pb^{2+} relative to the amount of DF present. (B) Comparison of calculated fits (lines) to observed data (circles/squares) at 335 nm for Pb^{2+}/Zn^{2+} titrations of DF (11 μM) (●) and CF (16.5 μM) (■). Inset: curves calculated assuming each Pb^{2+} binds five times more tightly than best fit values (dashed line above best fit line) and five times less tightly than best fit values (dashed line below best fit line) are shown at low equivalents of Pb^{2+}. These curves do not reproduce the data as well as the best fit (solid line) for each set of data and suggest that the $\beta_1^{Zn}/\beta_1^{Pb}$ and $\beta_2^{Zn}/\beta_2^{Pb}$ ratios (3.1 and 10, respectively) obtained from the best fits are robust.

metric ratio or whether a third weakly bound Pb^{2+} ion is present, the data from Pb^{2+} titrations of DF were fit to both 2:1 and 3:1 models. Comparison of fits to data for the two models provided in Figure 3C shows that the collected spectra are best fit by the 2:1 Pb^{2+}−DF model. This fit yielded the lower limit for $Pb^{2+}{}_2$−DF affinity of ${}^{DF}\beta_2{}^{Pb} \geq 5.0 \times 10^{17}\ M^{-2}$.

Pb^{2+}/Zn^{2+} Competition Experiments. The absolute affinities of Pb^{2+} for CF and DF were determined through metal-competition experiments in which the displacement of Pb^{2+} by Zn^{2+} was monitored by the resulting decrease in the Pb^{2+}-derived absorption bands. These Pb^{2+}/Zn^{2+} competition experiments yielded relative binding ratios for the two metals that were converted to the absolute affinity of Pb^{2+} for the CF and DF using the Zn^{2+} affinities for each domain.[76] Representative data collected from the competition experiment are provided for DF in Figure 4A. The data from CF competition experiments fit well to a model that included a colored 1:1 Pb^{2+}−CF species and the noncolored Zn^{2+}−CF species. A comparison of calculated fit to observed data for a representative titration fit using this model is included in Figure 4B. This model yielded ${}^{CF}\beta^{Pb}/{}^{CF}\beta^{Zn}$ of 0.32 for CF, which was converted to an absolute Pb^{2+} affinity of ${}^{CF}\beta^{Pb} = 6.4 \times 10^9\ M^{-1}$ using the previously reported affinity for Zn^{2+} (${}^{CF}\beta^{Zn} = 2.0 \times 10^{10}\ M^{-1}$).[76] The data obtained from DF Pb^{2+}/Zn^{2+} competition experiments were best fit using a model in which a Pb^{2+}−DF-Zn^{2+} species is explicitly included. Given that the NF and CF can bind metal independently,[76] both the spectrum of Pb^{2+}−CF and one-half of the spectrum of $Pb^{2+}{}_2$−DF (${}^{Pb_2DF}/_2$) were used as the basis spectra for Pb^{2+}−DF-Zn^{2+} in this model. These two different basis spectra were used in order to test the possibility that Zn^{2+} might preferentially displace Pb^{2+} from either the NF or CF sites, in which case the Pb^{2+}−CF basis spectra would differ in fit from that of (${}^{Pb_2DF}/_2$) if there were a spectroscopically detectable distinction between sites. Although both basis spectra yielded good fits to data and produced the same values of the equilibrium constants, plots of model error versus log β for each basis spectra revealed that only the Pb^{2+}−CF basis spectra were able to fit the collected data to a unique determined value. However, it is not clear whether this is due to preferential displacement of Pb^{2+} from the NF site or if the Zn^{2+} ion in Pb^{2+}−DF/Zn^{2+} alters the absorption spectrum of Pb^{2+}−DF/Zn^{2+} from that of Pb^{2+}−DF. A comparison of the calculated fit to observed data for this model is depicted in Figure 4B. This model yields relative binding ratios for the following Pb^{2+} and Zn^{2+} DF species: ${}^{DF}\beta_2{}^{Pb2}/{}^{DF}\beta_2{}^{Zn} = 0.10$ and ${}^{DF}\beta_2{}^{PbZn}/\beta_2{}^{Zn2} = 0.40$. Using the previously determined affinity of Zn^{2+} for DF (${}^{DF}\beta_2{}^{Zn2} = 6.3 \times 10^{20}\ M^{-2}$),[76] these binding ratios were converted to the following stability constants: ${}^{DF}\beta_2{}^{Pb2} = 6.3\ (\pm 6.3) \times 10^{19}\ M^{-2}$ and ${}^{DF}\beta_2{}^{PbZn} = 2.5\ (\pm 1.3) \times 10^{20}\ M^{-2}$.

Zn^{2+}/Pb^{2+} Competition Experiments. To determine whether Pb^{2+} could displace Zn^{2+} from these sites under physiologically relevant conditions, the reverse experiments in which Pb^{2+} was used to displace Zn^{2+} from CF and DF were also performed. A calculated titration using parameters obtained from Pb^{2+} forward (the Pb^{2+}−CF, $Pb^{2+}{}_2$−DF basis spectra) and Pb^{2+}/Zn^{2+} competition experiments (the affinity constants for Pb^{2+} CF and DF species) reasonably reproduced the observed titration data. (See Supporting Information.) Furthermore, these parameters provided a good estimate of the amount of Zn^{2+} displaced by Pb^{2+} (~17% of Zn^{2+}-GATA finger replaced by Pb^{2+}-GATA finger for the total concentration of GATA fingers in solution, 1 GATA finger for CF and 2 GATA fingers for DF, at a 30:1 Pb^{2+}/Zn^{2+} concentration ratio) at higher Pb^{2+} concentrations (30−40 molar equivalents Pb^{2+} per molar equivalent of GATA construct).

Effect of Pb^{2+} on In vitro GATA/DNA Binding. Having established the ability of Pb^{2+} to bind to CF and to displace Zn^{2+} as the bound cofactor when present in sufficient concentrations, we asked whether lead binding might have implications for the GATA/DNA interaction. This was assessed by challenging the Zn^{2+}−CF/DNA complex by addition of excess amounts of Pb^{2+} and assaying for DNA binding in a standard gel mobility shift assay. Consistent with the results described above that a large excess of Pb^{2+} is able to invade a Zn^{2+}−CF holoprotein and displace Zn^{2+} from a fraction of the available Zn^{2+}−CF protein, addition of a large (between 25- and 100-fold) excess of Pb^{2+} partially prevents formation of a CF/DNA complex (Figure 5). This effect on DNA binding suggests a possible mode of biological and toxicological action of Pb^{2+} that by being able to displace bound Zn^{2+}, Pb^{2+} subsequently inhibits the ability of the protein to bind to DNA.

Effect of Pb^{2+} on Transcriptional Activation in Yeast. To further probe the effects of Pb^{2+} on the GATA/DNA interaction in a cellular context, a yeast system was exploited in which GATA binding to DNA promotes transcription of the LacZ gene,

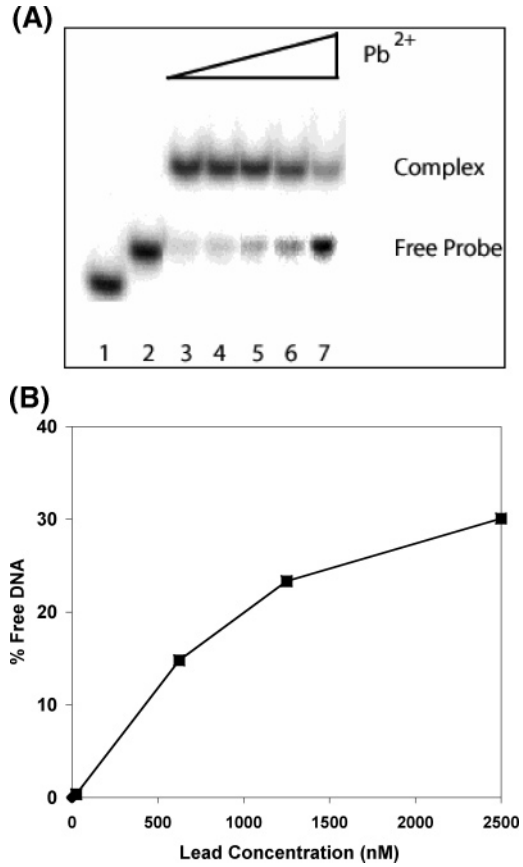

Figure 5. Gel mobility shift assay of GATA incubated with lead. (A) Change in mobility of AGATA DNA when incubated with Zn^{2+}−CF treated with a varying concentration of lead. Lane 1, ssDNA; 2, dsDNA; 3, + Zn^{2+}−CF, 0 nM Pb^{2+}; 4, + Zn^{2+}−CF, 25 nM Pb^{2+}; 5, + Zn^{2+}−CF, 625 nM Pb^{2+}; 6, + Zn^{2+}−CF, 1.25 μM Pb^{2+}; 7, + Zn^{2+}−CF, 2.5 μM Pb^{2+}. In all experiments, ^{32}P-labeled AGATA DNA and Zn^{2+}−CF were both at 25 nM. Positions of the free probe and CF/DNA complex are shown. (B) Plot of percentage-free DNA over the range of lead concentrations. Densities of the free and complexed signals in A were measured using ImageQuant.

which can be monitored conveniently using an assay for β-galactosidase activity. Transcriptional activation in this system is dependent on a functional CF domain fused to the yeast GAL4 transcription activation domain and on the presence of consensus GATA-binding sites in the promoter driving LacZ expression. Addition of Pb^{2+} in growing cultures of these cells results in inhibition of β-galactosidase activity, as judged by measurement of formation of yellow *o*-nitrophenol by hydrolysis of *o*-nitrophenylgalactoside substrate. When Pb^{2+} was added to 100 μM in the liquid culture medium in the GATA-activated system, β-galactosidase activity was reduced to about 50% of that observed in the absence of Pb^{2+} (Figure 6). In contrast, addition of Pb^{2+} to control yeast bearing the p53/GAD activator protein and p53-drive LacZ reporter gene resulted in minimal loss of activity over the range of Pb^{2+} added. The p53 DNA binding activity provides a useful comparison as p53 possesses a Cys_3-His Zn^{2+} site that is also essential for DNA binding.[82,83] However, lead-binding studies on model systems predicted that the Cys_3His site of p53 should be less sensitive to Pb^{2+} than the Cys_4 site in GATA proteins.[19] The results observed for this system (that the p53-mediated transcription is not altered at

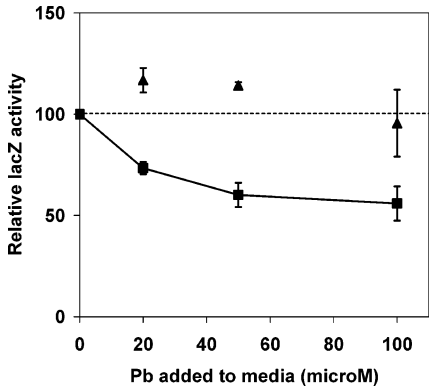

Figure 6. β-Galactosidase (LacZ) activity in yeast cells was assayed by ONPG hydrolysis in permeablized cells. Activities were determined for yeast expressing LacZ by GATA (■) or by p53 (control; ▲) mediated promoter binding. Measurements are replicates of four (GATA) or three (p53) separate cultures for each concentration of Pb^{2+}, given as activity normalized to culture density, relative to activity in the absence of added Pb^{2+} as 100%. Error bars represent standard error for each set of data.

concentrations of Pb^{2+} that result in a significant decrease in GATA-mediated transcription) are consistent with the idea that Pb^{2+} exhibits an inherent thermodynamic preference for Cys_4 zinc sites.

Addition of Pb^{2+} to a concentration of 1000 μM was observed to dramatically reduce yeast growth rate and thus appeared to have more general effects on endogenous yeast proteins as well as the GATA transgene (data not shown). Analysis of washed yeast pellets from Pb^{2+}-grown cultures by ICP−MS reveals that the yeast accumulate Pb^{2+} in a dose-dependent fashion (see Supporting Figure 4).[84] Taken together with the spectroscopic and gel shift analyses described above, the yeast one-hybrid experiments suggest that the ability of Pb^{2+} to interfere with normal Zn^{2+}-bound GATA is due to an equilibrium competition between the two metal ions for the GATA metal-binding sites.

Discussion

Given recent evidence that Pb^{2+} prefers cysteine-rich zinc-binding sites in proteins and the observation that Pb^{2+} affects many processes that are regulated by GATA proteins, including neurological development and cardiovascular disease, the Cys_4 Zn^{2+} sites of GATA proteins are a logical potential target for Pb^{2+}. Here, we have conducted rigorous metal-binding studies on two GATA constructs, the C-terminal GATA finger from chicken GATA-1 (CF) and the double-finger domain from human GATA-1 (DF), to address the following questions. (1) Does Pb^{2+} form tight complexes with the cysteines in the zinc-binding sites of GATA? (2) What is the stoichiometry of the Pb^{2+} complexes with the CF and DF constructs? (3) Is Pb^{2+} able to displace Zn^{2+} from these sites under physiologically relevant conditions? (4) Does Pb^{2+} interact with GATA in a biologically significant way by affecting DNA binding?

The interaction of Pb^{2+} with CF and DF was monitored spectroscopically using intense bands that emerge as Pb^{2+} coordinates to the cysteines in the GATA zinc-binding site. Pb^{2+} binds to CF in a 1:1 stoichiometric ratio, the same as that observed for Co^{2+} and Zn^{2+}.[72,76] Pb^{2+} saturates DF between 2 and 2.4 molar equiv of Pb^{2+} to DF. Given that Pb^{2+} has been

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Table 1. Binding Constants for Pb^{2+} and Selectivity over Zn^{2+} ($\beta 1^{Zn}/\beta 1^{Pb}$) for Several Structural Zinc-Binding Domains (pH 7.0)

single zinc sites	type of site	β_1^{Pb} (M^{-1})	β_1^{Zn} (M^{-1})	$\beta_1^{Zn}/\beta_1^{Pb}$	refs
CP-CCCC	Cys_4	2.6×10^{13}	9.0×10^{11}	0.034	19, 24
CP-CCHC	$Cys_2HisCys$	1.3×10^{10}	3.1×10^{11}	24	19, 24
CP-CCHH	Cys_2His_2	5.0×10^{9}	1.8×10^{11}	9	19, 24
HIV-CCHC	$Cys_2HisCys$	3.3×10^{9}	1.4×10^{10}	4.3	19, 24
CF	Cys_4	6.4×10^{9}	2.0×10^{10}	3.1	this paper
double zinc sites	**type of site**	β_2^{Pb} (M^{-2})	β_2^{Zn} (M^{-2})	$\beta_2^{Zn}/\beta_2^{Pb}$	
DF	Cys_4	6.3×10^{19}	6.3×10^{20}	10	this paper

observed to form stable trigonal thiol complexes,[17,85,86] the possibility of a third Pb^{2+} binding weakly to DF was considered. However, the data collected from Pb^{2+} titrations of DF could not be fit well to a 3:1 model (see Figure 3C), whereas a 2:1 model provided an excellent fit to these data with ${}^{DF}\beta_2^{Pb2} \geq 5.0 \times 10^{17}\ M^{-2}$.

The absolute values of the stability of Pb^{2+} complexes with CF and DF were determined through competition experiments in which Zn^{2+} was used to displace bound Pb^{2+} from the domains. The CF data fit well to a model that included 1:1 $Pb^{2+}-CF$ and $Zn^{2+}-CF$ species and yielded as the absolute affinity of ${}^{CF}\beta_1^{Pb} = 6.4\ (\pm 2.0) \times 10^9\ M^{-1}$ for Pb^{2+} binding to CF. The DF data were best fit by a model that explicitly included both 2:1 $Pb^{2+}-DF$ and a 1:1:1 $Pb^{2+}-DF/Zn^{2+}$ species and yielded ${}^{DF}\beta_2^{Pb2} = 6.3\ (\pm 6.3) \times 10^{19}\ M^{-2}$ and ${}^{DF}\beta_2^{PbZn} = 2.5\ (\pm 1.3) \times 10^{20}\ M^{-2}$.

These experiments show that while Pb^{2+} does not form complexes with CF or DF as tightly as Zn^{2+}, Pb^{2+} does bind with high affinity to both these domains (${}^{CF}\beta_1^{Zn} = 2.0\ (\pm 1.3) \times 10^{10}\ M^{-1}$ vs ${}^{CF}\beta_1^{Pb} = 6.4\ (\pm 2.0) \times 10^9\ M^{-1}$, and ${}^{DF}\beta_2^{Zn} = 6.3\ (\pm 2.5) \times 10^{20}\ M^{-2}$ vs ${}^{DF}\beta_2^{Pb} = 6.3\ (\pm 6.3) \times 10^{19}\ M^{-2}$). The high affinity of Pb^{2+} for CF, given by ${}^{CF}\beta_1^{Pb} = 6.4\ (\pm 2.0) \times 10^9\ M^{-1}$, is within the range of values reported for other structural zinc-binding domains by Godwin and co-workers[19] (see Table 1). For example, the $Pb^{2+}-CF$ affinity is on the same order of magnitude as the affinity of Pb^{2+} reported for the HIV-CCHC nucleocapsid protein, given by ${}^{HIV\text{-}CCHC}\beta_1^{Pb} = 3.0 \times 10^9\ M^{-1}$.[19] The affinity of Pb^{2+} for both CF and the HIV-CCHC domain is $\sim 3-4$ orders of magnitude lower than ${}\beta_1^{Pb} = 2.6 \times 10^{13}\ M^{-1}$ reported for the model peptide CP-CCCC.[19] This observation is consistent with the observation that the consensus peptides typically bind Co^{2+} and Zn^{2+} more tightly than peptides that correspond to naturally occurring sequences with the same metal-binding residues, presumably this reflects the fact that the metal-binding site of the consensus peptides is, by definition, "optimized".[24,76,87]

The fact that the 1:1:1 $Pb^{2+}-DF/Zn^{2+}$ species is required for a good fit indicates that metal binding can occur at the two sites independently, which is in good agreement with the results from previous metal-binding studies using Co^{2+} and Zn^{2+}.[76] For GATA-1, the CF domain is both necessary and sufficient for DNA binding and transactivation at GATA sites.[73,88,89] Although initial experiments suggested minor roles for the NF domain of GATA-1, such as stabilizing binding at certain sites and interacting with protein partners,[88,89] subsequent work showed the NF domain is required for GATA-1−DF binding at a subset of GATA sites known as GATApal.[74] Additionally, the NF domain of GATA-1 binds stably to GATC DNA elements and interacts with these GATC elements as part of the DF domain.[74,75,90,91] Furthermore, the NF domain in the GATA-1−DF was shown to modulate the DNA binding and transactivation compared to that of the CF domain alone, in conjunction with the composition and spacing of a double GATA DNA site, such that the GATA-1−DF domain has its own binding and transactivation profile not characteristic of a single CF domain.[75,91] Evidence that the effects of the NF domain in modulating the CF domain in DF domain action are physiologically relevant is provided by rescue experiments in GATA-1 knockout mice in which the DF domain of GATA-1 was absolutely required for definitive red blood cell production while the CF domain of GATA-1 rescued only the very earliest stages of red blood cell development.[92] Given that both the NF and CF domains of GATA-1 are important for proper function, this further suggests that Pb^{2+} could interfere with GATA action even if only one Zn^{2+} was displaced from GATA.

To confirm that tight Pb^{2+} binding to CF and DF corresponded to Pb^{2+} having the ability to displace Zn^{2+} from these domains, the reverse of the above competition experiment was performed in which Pb^{2+} was titrated into the Zn^{2+}-bound GATA constructs. In this series of experiments, Pb^{2+} was able to displace Zn^{2+}, and the resulting absorption spectrum ceased to change within minutes at 37 °C. This result indicates that metal exchange occurs on a physiologically relevant time scale. Although Zn^{2+} is the thermodynamically preferred metal for both CF and DF, Pb^{2+} still displaces a significant percentage of Zn^{2+} from GATA sites for both constructs ($\sim$17% Pb^{2+}-GATA finger per total number of GATA sites, i.e., 1 GATA site for CF and 2 GATA sites for DF, at a 30:1 Pb^{2+}/Zn^{2+} concentration ratio). These data suggest that GATA proteins are likely targets for Pb^{2+} particularly in tissues in which Pb^{2+} is sequestered (e.g., the brain and the heart).[93−96]

If GATA zinc-finger domains are indeed targets of Pb^{2+} toxicity in vivo, it would be necessary for the interaction of Pb^{2+} with the protein to perturb the biological function of normal GATA proteins, presumably either by impeding GATA's ability to bind DNA or, once bound, by abrogating its transcription activation function, or both. The NMR-derived structure of the C-terminal domain of chicken GATA-1 showed that the metal-binding cysteines must assume a tetrahedral coordination geometry to stabilize the discrete GATA secondary structure. Despite forming tight complexes with CF and DF, Pb^{2+} is unlikely to stabilize the same conformation of GATA domains

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because Pb^{2+} is unlikely to assume the regular tetrahedral coordination geometry required for GATA folding.[17,70,71,81,85,86] In several cases that have been investigated previously, Pb^{2+} has been shown to bind tightly to structural zinc-binding domains, but not promote proper folding and activity of the protein. Godwin and co-workers used NMR and CD spectroscopy to demonstrate that the Pb^{2+}-HIV-CCHC had a structure different than that of Zn^{2+}-HIV-CCHC.[19] Pb^{2+}-based disruption of the DNA-binding activity of the structural zinc-binding domain has also been observed for recombinant human Sp1 and TFIIIA, both of which contain a Cys_2His_2 zinc-finger domain.[18,21] Pb^{2+} has been found to alter the DNA-binding activity of these proteins in other ways as well; Zawia and co-workers report that Pb^{2+} can actually stimulate binding of a synthetic Sp1 peptide to DNA.[21] Here, when Pb^{2+} was added to a solution containing apo-CF, no shift was observed in the methyl resonances of the 1D ^1H NMR spectrum and no increase was observed in the chemical shift dispersion of the amide proton region of the spectrum.[97] The lack of these changes, which do occur upon addition of zinc[70] and are generally considered indicative of metal binding in structural zinc-binding peptides,[98−100] suggests that Pb^{2+}−CF is not folded in solution. Taken together, these studies show that Pb^{2+} bound to structural zinc-binding domains alters both their structure and their action. The studies reported herein indicate that Pb^{2+} bound to GATA proteins disrupts GATA protein function both in vitro in gel shift assays and in vivo in the yeast assay, resulting in both cases in a reduction of DNA binding. Because excess amounts of Pb^{2+} can only partially compete away Zn^{2+} from holo-GATA CF, it seems likely that the residual binding activity observed in vitro and in vivo at the higher concentrations of added Pb^{2+} represents a fractional population of Zn^{2+}-GATA which has escaped disruption by Pb^{2+}. However, it remains possible that in these assays that Pb^{2+} has substantially replaced Zn^{2+} and that the resulting Pb^{2+}-GATA retains a measurable but weakened ability to bind DNA. In either event, the results reported herein established that interference by Pb^{2+} inhibits GATA action at the level of DNA binding.

Conclusions

Here, we have provided detailed metal-binding studies that reveal that Pb^{2+} forms tight complexes ($^{CF}\beta_1^{Pb} = 6.4\ (\pm 2.0) \times 10^9\ M^{-1}$ and $^{DF}\beta_2^{Pb2} = 6.3\ (\pm 6.3) \times 10^{19}\ M^{-2}$) with the Cys-rich Zn^{2+} site in GATA proteins, with one Pb^{2+} ion binding to each native Zn^{2+} site. In addition, we demonstrated that Pb^{2+} can displace Zn^{2+} from these sites under physiologically relevant conditions to disrupt GATA/DNA interaction. Given that Pb^{2+} exhibits different coordination geometries than does Zn^{2+}, it is likely that the Pb^{2+} inhibits GATA function by altering the structure of the zinc-binding domains. Because of the modular nature of the composite GATA-DF domain, Pb^{2+} replacement of Zn^{2+} at a single site as observed here would be expected to alter action of full-length GATA proteins in the tissues in which they are active. Therefore, we propose that Pb^{2+} would have the most deleterious effects upon the activity of GATA proteins in those tissues that are known to accumulate significant amounts of Pb^{2+} in vivo. Given the important role that GATA proteins play in neurological development and hematopoeisis, these data suggest that the effect of Pb^{2+} on GATA-mediated gene expression in developing organisms needs to be explored. In addition, the accumulating evidence that GATA-4 is essential to cardiac hypertrophy and the development of heart disease suggest that GATA-4 should be investigated as a target for Pb^{2+} action in the heart in adults.

Acknowledgment. This work was supported by NIH Grant 1 R01 GM58183-01A1 (H.A.G.), American Cancer Society Grant RPGLBC-100183 (J.G.O.), and NIH Grant R15HL66009-01A1 (M.P.). Hilary Arnold Godwin is a Howard Hughes Medical Institute Professor and a recipient of a Camille and Henry Dreyfus New Faculty Award, a Burroughs Wellcome Fund New Investigator Award in the Toxicological Sciences, an NSF CAREER Award, a Sloan Research Fellowship, and a Camille Dreyfus Teacher-Scholar Award. Ultraviolet-visible spectra were collected at the Keck Biophysics Facility at Northwestern University [http://www.biochem.northwestern.edu/Keck/keckmain.html]; ICP−MS data were collected in the Analytical Services Laboratory in the Department of Chemistry at Northwestern University.

Supporting Information Available: Absorption spectra collected for direct Pb^{2+} titration of CF and Zn^{2+} competition titrations of CF; comparison of calculated and observed titration curves for the Zn^{2+}/Pb^{2+} competition experiment using DF. Methods and data are presented for uptake of Pb^{2+} into *S. cerevisiae*. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0464544

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