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The ^1H NMR Structure of Bovine Pb^{2+} - Osteocalcin and Implications for Lead Toxicity

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SUMMARY

Structural information on the effect of Pb^{2+} on proteins under physiologically relevant conditions is largely unknown. We have previously shown that low levels of lead increased the amount of osteocalcin bound to hydroxyapatite (BBA 1535:153)). This suggested that lead induced a more compact structure in the protein. We have determined the 3D structure of Pb^{2+} -osteocalcin (49 amino acids), a bone protein from a target tissue, using ^1H 2D NMR techniques. Lead, at a stoichiometry of only 1:1, induced a similar fold in the protein as that induced by Ca^{2+} at a stoichiometry of 3:1. The structure consisted of an unstructured N terminus and an ordered C-terminal consisting of a hydrophobic core (residues 16–49). The genetic algorithm –molecular dynamics simulation predicted the lead ion was coordinated by the Gla 24 and Gla 21 residues. It is proposed that mineral binding occurs via uncoordinated Gla oxygen ions binding to calcium in hydroxyapatite. A comparison of Pb^{2+} - and Ca^{2+} -osteocalcin suggests Pb^{2+} , at a lower stoichiometry, may induce similar conformational changes in proteins and subsequent molecular processes normally controlled by calcium alone. This may contribute to a molecular mechanism of lead toxicity for calcium binding proteins. Lead exposure may alter the amount of mineral bound osteocalcin and contribute to abnormal bone remodeling.

Keywords

Osteocalcin; NMR; Pb^{2+} ; Lead Toxicity

INTRODUCTION

Lead toxicity remains a major public health problem in the United States. In 2004, elevated blood lead levels ($> 0.5 \mu\text{M}$, $10 \mu\text{g/dL}$) were reported in approximately 434,000 people (1). Bone is the major reservoir of lead in the body, accounting for 75% of total body lead in

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affected children and over 90% in adults [2,3]. Elevated bone lead has been found in children exposed to high environmental levels [4] and in occupationally exposed adults [5,6]. Even after lead exposure has ceased, the normal process of bone turnover results in the continuous release of lead into the circulation and contributes to detrimental effects of lead on soft tissue in normal and pathological states [7,8]. Lead also has direct negative effects on bone. Chronic low level lead exposure results in decreased bone mineral density in adults and alterations in growth in children (9,10). Animals exposed to lead show reduced bone density (11), and delayed fracture healing (12). Several studies have indicated that lead can effect both bone formation and resorption (13–15). At the cellular level lead alters the activity of osteoclasts (16) and perturbs intracellular Ca^{2+} concentrations and bioenergetics in osteoblasts (17,18).

At the molecular level, the effects of lead are not fully understood. Very few structural studies have been reported, especially for Ca^{2+} binding proteins. Lead has been shown to bind with high affinity and displace calcium in calmodulin, calbindin and troponin C [19,20]. It has been shown to functionally substitute for calcium in the activation of protein kinase C [21] and calmodulin dependent phosphodiesterase [19] and myosin light chain kinase [22]. The x-ray crystal structure of Pb^{2+} -calmodulin demonstrated that 3 of the Ca^{2+} binding sites in calmodulin were occupied with Pb^{2+} while the fourth was partially occupied. This induced a structure similar to Ca^{2+} -calmodulin in the C-terminal domain but with more interhelical flexibility in the N-terminal domain (23). There is a need for more structures of Pb^{2+} complexed proteins to understand its effect at the molecular level.

We have previously reported the effect of Pb^{2+} on the Ca^{2+} binding protein osteocalcin, an abundant noncollagenous matrix protein found in bone [24,25] and dentin [26]. Important structural features of osteocalcin include 3 γ -carboxyglutamic acid residues (Gla) which bind Ca^{2+} in solution and in the mineral phase [27]. At a stoichiometry of 3:1, Ca^{2+} induces a compact structure which facilitates binding of osteocalcin to hydroxyapatite [28,29,30]. Our circular dichroism studies showed that lead binds osteocalcin with an affinity over 4 orders of magnitude tighter than does calcium and induces a similar conformational change but at a 1:1 ratio [29]. In addition, in the presence of Pb^{2+} the amount of osteocalcin which binds to hydroxyapatite *in vitro* was increased, leading us to hypothesize that Pb^{2+} may induce a more compact structure in the protein [29]. In order to confirm this hypothesis, and to investigate the effect of Pb^{2+} on another Ca^{2+} binding protein, we determined the structure of Pb^{2+} -complexed osteocalcin, at physiologically relevant conditions, using ^1H NMR, and compared it to the structure of Ca^{2+} -osteocalcin.

MATERIALS AND METHODS

Sample Preparation

Bovine osteocalcin was used for the studies described here. It has 90% sequence identity to human osteocalcin and it is more soluble at millimolar concentrations needed for 2D NMR studies. Native osteocalcin was purified from bovine bone powder as previously described [31]. Purity of the protein was determined by polycacrilimide gel electrophoresis, reverse phase HPLC, amino acid analysis and electrospray mass spectrometry. This material was used exclusively for 1 and 2D NMR data for structural calculations. The 2D preliminary

experimental data were also collected on synthetic bovine osteocalcin containing gla at positions 17, 21, and 24. This material was used only for optimizing solution conditions and temperatures for 2D NMR spectra collection and was characterized as above. It was synthesized using solid phase peptide synthesis methodology on an ABI 433 peptide synthesizer using Fmoc protocols as previously described [29,32]. The synthetic protein was found to give similar CD, 1D, and 2D NMR spectra and to bind to hydroxyapatite in a similar manner to the osteocalcin prepared from calf bone.

We have previously shown that the CD titration data of osteocalcin with Pb^{2+} can be fit to one binding site at protein concentrations of 0.25–.50 μM at pH 7 [29]. To show this with NMR, an NMR titration at different lead to osteocalcin ratios was conducted in 20 mM NaCl, 75 μM osteocalcin and $\text{Pb}(\text{NO}_3)_2$ at a lower pH to eliminate solubility problems at the higher protein concentration, at increased lead to protein ratios (similar to what was observed with higher concentrations of calmodulin [23]). To solve the high resolution structure of Pb^{2+} -osteocalcin the NMR spectra were collected in H_2O solutions containing 1.70 mM protein, 1.70 mM $\text{Pb}(\text{NO}_3)_2$, 20 mM NaCl and 10% $^2\text{H}_2\text{O}$ at a pH of 6.8 and temperatures of 37, 27 and 22 $^\circ\text{C}$. For samples collected in $^2\text{H}_2\text{O}$, the sample was lyophilized from $^2\text{H}_2\text{O}$ several times and then resuspended in 99.96% $^2\text{H}_2\text{O}$. The pH was adjusted before each experimental run and found to be stable during the run. It is possible to calculate (using the concentrations of 1.70 mM osteocalcin, 1.70 mM $\text{Pb}(\text{NO}_3)_2$ and a K_d of 85 nM) that the free Pb^{2+} is approximately 12 μM . Therefore 99.3% of the protein is bound to lead with our experimental conditions.

Analytical Ultracentrifugation

Sedimentation equilibrium experiments were performed on apo- and Pb^{2+} -bound osteocalcin in order to determine the oligomerization state of the protein as previously described in our study of the Ca^{2+} -bound protein [28].

NMR Spectroscopy

All NMR spectra were collected on a Bruker DRX 600 MHz spectrometer. The double gradient echo method of Hwang and Shaka [33] was used for water suppression in all 2D experiments. All 2D data sets in H_2O were collected at temperatures of 310, 300 and 295 K with either TPPI or States-TPPI used for quadrature detection in the second dimension [34,35]. The data were processed using NMRPipe [36] and extended using linear prediction and zero filling. The data were analyzed using NMRView [37].

All proton resonances were assigned using standard procedures developed by Wüthrich [38]. Intraresidue proton resonance assignments were made using DQF-COSY and both short (17 ms) and long (60 ms) mixing time TOCSY experiments using 640 t1 increments [39]. A natural abundance ^{13}C -H HSQC experiment was collected with 160 t1 increments to obtain C_α and C_β chemical shifts. Sequential assignments and distance constraints were obtained from NOESY spectra collected using a 150 ms mixing time and 640 t1 increments. TOCSY and NOESY spectra were also collected in $^2\text{H}_2\text{O}$ to obtain spin system assignments close to the H_2O resonance as well as to distinguish between aromatic and amide protons.

Structure Calculation

NOE crosspeak intensities were classified as strong, medium and weak and were converted to distance restraints of 1.8–2.8, 1.8–3.4 and 1.8–5.5 Å respectively. Distance corrections for pseudoatoms were calculated as previously described [28]. Dihedral angle restraints were obtained from H_{α} , $^{13}C_{\alpha}$, and $^{13}C_{\beta}$ chemical shift analysis using the program Talos [40]. Amide proton temperature coefficients were calculated from the proton chemical shift change from 15–37 °C and amide protons involved in hydrogen bonds were determined as reported previously [28]. Initial structures were calculated using NOE constraints, dihedral angle constraints, and H_{α} and $^{13}C_{\alpha}$, chemical shifts by simulated annealing and torsional angle dynamics as applied in the program CNS 1.1 [41]. Final structures were then calculated using CNS 1.1 with hydrogen bonds as additional constraints. The 10 lowest energy structures were evaluated with AQUA and PROCHECK-NMR [42] and displayed using MOLMOL [43].

Identification of the Positions of Pb^{2+} Ions in Osteocalcin

The previously described genetic algorithm-molecular dynamics method [44] was used to place the lead ion in osteocalcin and also used for further energy minimization and refinement of the metal-bound structure. The initial structures used were the 10 best conformers determined by NMR of the Pb^{2+} -osteocalcin complex. The genetic algorithm was used to determine potential lead binding sites by searching all O-O midpoints which were within 7 Å of each other. The genetic algorithm [44] was subsequently used to search for the one Pb^{2+} binding site on each conformer. Multiple independent runs of searches were carried out for each conformer and the site with the lowest potential energy, determined within the program Amber [45], was taken as the site for the conformer. The energies were converged (the same lowest energy between runs). Among the 10 conformers, the most frequently occurring site had the same two Glu residues coordinating the Pb^{2+} in a similar fashion. This result suggests that it is reasonable to average the 10 initial NMR structures and apply the genetic algorithm on the average structure. The average structure and the predicted Pb^{2+} site was further refined using a molecular dynamics simulation with Particle-Mesh Ewald [46] to accommodate long-range electrostatics and determine the best position of the Pb^{2+} coordinating residue side chains and the lead ion. Briefly, this simulation was accomplished by solvating the protein in a 40 Å box of water. Initially, the H_2O , Pb^{2+} and Na^+ (counterions for charge neutralization) were energy minimized at constant volume as previously described [44]. Since the NMR constraints had determined the backbone conformation a Particle Mesh Ewald molecular dynamics simulation was performed on the side chains of the lead coordinating residue and on the Pb^{2+} , Na^+ and water molecules only for 350 ps at constant pressure and with fixed backbone.

RESULTS

We have previously shown that CD titration data with lead can be fit to one binding site at low protein concentrations of (0.25–0.5 μM) at physiological pH [29]. It was found that a ratio of Pb^{2+} /osteocalcin of 1 induced the maximal conformational change in the protein as observed by CD. Figure 1a shows an NMR titration of the aromatic and NH proton region of apo-osteocalcin with Pb^{2+} producing Pb^{2+} /osteocalcin ratios of 1, 2 and 3. The shifted

tyrosine resonances are marked. These resonances were shown to be involved in the formation of a hydrophobic core in our previously solved NMR structure of Ca^{2+} -osteocalcin [28]. It is clear that the major conformational change in the 1D NMR spectrum, the formation of a hydrophobic core, occurs at a Pb^{2+} /osteocalcin ratio of 1 and there is little change with increasing lead to protein ratios. We have previously shown that a ratio of Ca^{2+} /osteocalcin of 3 induced the maximal conformational change as observed in the 1D spectrum of osteocalcin [28]. Figure 1b shows a comparison of 1D NMR spectra of apo-osteocalcin and at ratios of calcium/osteocalcin of 3 and lead/osteocalcin of 1. Addition of Pb^{2+} at a ratio of only 1 shifts the aliphatic protons of valine, leucine and isoleucine as well as the ring protons of tyrosine (lines in Figure 1b) similarly to that induced by a ratio of Ca^{2+} /osteocalcin of 3. The Glu β -proton resonances were also shown to be altered with Pb^{2+} addition. These resonances have previously been shown to be Ca^{2+} -coordinating residues [28]. This suggests that the Glu β -proton resonances may also be a coordination site for Pb^{2+} . These spectra show that lead can induce similar alterations in resonances from hydrophobic residues in osteocalcin as calcium but at a much lower stoichiometry. Figure 1c shows 1D NMR spectra of the protein at a Ca^{2+} /osteocalcin ratio of 1 (lower spectrum) and at a Pb^{2+} /osteocalcin ratio of 1 (upper spectrum). In contrast to Pb^{2+} , it is clear that in the 1D spectrum, the maximal conformational change in the protein does not occur at a Ca^{2+} /osteocalcin ratio of 1. In order to determine differences or similarities in the detailed structures as well as the coordination site of the Pb^{2+} ion, we collected 2D NMR data and solved the three dimensional structure of Pb^{2+} -osteocalcin.

Proton resonance assignments were made using a combination of two-dimensional DQF-COSY, short and long mix TOCSY experiments. Sequential assignments were made using the NH- α H region as well as the amide-amide region of the NOESY spectra collected in H_2O . NOESY experiments in $^2\text{H}_2\text{O}$ were used to identify interactions between aromatic side chains and aliphatic protons. Figure 2 shows long-range contacts observed in the NOESY spectrum. Long range NOEs between the aromatic side chain protons of Y42 and Y46 with side chain protons of R20, C23 and V22 are shown. The shifted methyl and aromatic protons in the 1D spectrum are due to some of these interactions.

Structure Calculation

A total of 552 NOE distance constraints were used in the calculation of the structure of Pb^{2+} -osteocalcin, 65 of which were intraresidue, 230 were short range, 169 were medium range and 88 were long range. There were also 78 dihedral constraints, 34 chi angle constraints, 45 H_α , 38 C_α , and 26 C_β chemical shifts used in the calculation. The distribution of NOE constraints, characteristic NOE patterns, and H_α and C_α chemical shifts along the sequence of osteocalcin are shown in Figure 3. Longer range and medium range NOE's are located predominantly within residues 16-49 in the sequence. The absence of longer range NOE's within the first 15 amino acids suggests a lack of structure in this area of the molecule. Backbone NOE patterns along the sequence are shown in the lower half of Figure 3. Backbone NOE patterns indicative of helices and turns (intense NH-NH NOEs, weaker intensity, nonsequential NOE patterns ($d_{\alpha\text{N}}(i,i+3)$, $d_{\alpha\beta}(i,i+3)$, $d_{\alpha\text{N}}(i,i+4)$ and $d_{\text{NN}}(i,i+2)$) are observed only from residues 22-47 in the sequence. Figure 3 also shows secondary proton chemical shift values representing the difference between the observed αH chemical shift

from that reported in a random coil conformation. Positive values for ^1H and ^{13}C secondary shifts are also observed in areas of α -helical structure [47]. Figure 3 also shows elevated ^1H and ^{13}C secondary shift values for residues 22-47. Almost all of the measured temperature coefficients gave values of less than 5 for NH protons in residues 16-47 indicating folded regions in the molecule and the probability that these protons are hydrogen bonded. The calculated structures were consistent with 5 hydrogen bonds marked in Figure 3 with filled circles. Overall the data indicates 1 structured area and several loose turns in the molecule within residues 16-47 and residues 1-15 are unstructured.

Structure Calculation and Description

The structures were calculated using torsion angle dynamics and refined using simulated annealing and energy minimization using the program CNS 1.1 [41]. The N-terminus (residues 1–16) was found to be largely unstructured, flexible and to have no fixed orientation with respect to the rest of the molecule. Figure 4A shows a bundle consisting of the superposition of the backbone atoms of the structured region (residues 17-47) for the 10 lowest energy structures. The total backbone rmsd for this region is 2.00 Å and there were no distance restraint violations greater than 0.5 Å and no dihedral angle restraint violations greater than 5°. This indicates that the structures fit the NMR data well and that one conformation can be defined for the molecule. Since there was only 1 Pb^{2+} ion bound to the molecule it is possible that Gla17 is not involved in the coordination. Superimposing the backbone atoms of residues 21- 47 gives an improved rmsd of 1.70 Å as shown in Figure 4B. The smaller rmsd from residues 21-47 suggests that Pb^{2+} may be coordinated to residues 21 or beyond causing less flexibility and a more defined structure in these regions. Figure 4C shows the one structured area common to all 10 models. Superposition of residues 27-34 gives an rmsd of 0.57 Å. Superposition of the Gla region (Gla24 and 21) with the side chains in residues 21-25 gives an rmsd of 2.616 Å as shown in Figure 4D.

A low energy structure of Pb^{2+} -osteocalcin is shown in Figure 5A with the previously solved NMR structure of Ca^{2+} -osteocalcin given for comparison in Figure 5B [28]. As can be seen in Figure 5A there is an unstructured N terminus from residues 1-15 and a structured region from residues 16-49 which contains the region of secondary structure. Analysis of ϕ , θ backbone angles allowed the areas of secondary structure within the structured region (16-49) to be classified [48]. Residues 27–30 form a type III turn and residues 31–34 form a 3_{10} helix. A couple of structures within the family of structures show an α -helical structure within residues 41-44. Residues 21–26 form loose turns with helical characteristics and a disulfide bond between $\text{C}_{23} - \text{C}_{29}$ separates this region from the 3_{10} helical region. The secondary structural areas are stabilized by hydrogen bonds. The tertiary structure of the molecule consists of a C-terminal core (residues 16-49) formed by various long range and hydrophobic side chain interactions such as those between Leu 16 and Phe 45, and Tyr 42 and Tyr 46 with Val 22 and Cys 23 as shown in Figure 5C.

Comparison of Figure 5A, Pb^{2+} -osteocalcin, with 5B, Ca^{2+} -osteocalcin shows that lead binding to the protein with a stoichiometry of 1:1 induces a similar fold in the protein as does calcium which binds with a stoichiometry of 3:1. There is an unstructured N-terminus and a similar hydrophobic core formed from similar long range hydrophobic interactions.

However, lead induces less secondary structural elements in the protein as compared to calcium. The Ca^{2+} -osteocalcin structure contains two type III near reverse turns within residues 20–23 and 23–26, and 2 α -helical segments within residues 27–35 and 41–44. The lead structure has only one area of secondary structure which is a 3_{10} helix and type III turn within residues 27–34. The increased number of Ca^{2+} ions (3) bound to the structure may serve to neutralize the charge and stabilize these helical regions explaining the decreased secondary structure seen in Pb^{2+} -osteocalcin.

Docking of Pb^{2+} in Osteocalcin

There is one Pb^{2+} ion bound to osteocalcin in this study. We used the genetic algorithm-molecular dynamics simulation method, applied to other NMR structures of Ca^{2+} binding Gla containing peptides and proteins including Ca^{2+} -osteocalcin [28,44,49], to identify the position of the lead ion. All 10 conformers in the family of structures were searched with the genetic algorithm and the most frequently occurring coordinated structure had the lead ion coordinated by side chains of Gla 24 and Gla 21. The orientation of the N-terminal unstructured region of the protein is in a different position in each conformer, had no sole coordination site and was predominantly uncoordinated in all 10 conformers. Thus, an average structure was created from residues 16–49 and the genetic algorithm identified the lead ion position, which gave the lowest energy structure. This structure, along with 5 Na^+ ions to balance the charge, was solvated in water and subjected to a molecular dynamics simulation where the Gla side chains and the ions have their geometries and energy optimized. This procedure resulted with the Pb^{2+} ion coordinated to the side chains of the Gla 24 and Gla 21 residues as shown in Figure 6. The lead ion was considered to be coordinated to an oxygen if the distance between the two ions was 3 Å or less. The lead ion was coordinated by the side chain oxygen ions OE3 and OE4 of Gla 24, and the side chain oxygen ions OE1 and OE4 of Gla 21 and oxygens from 3 water molecules.

The Pb^{2+} coordinated structure (residues 16–49) is shown in Figure 6. It can clearly be seen that it takes the binding of only one Pb^{2+} ion per osteocalcin molecule to induce a similar fold in the protein (hydrophobic core formation at the C-terminal end and unstructured N-terminus), as that which requires 3 calcium ions. The 3 Ca^{2+} ion binding sites were found to include coordinating residues Asp 30 and Gla 24, Gla 24 and Gla 21, and Gla 21 and Gla 17 (30). Thus, lead does not have a unique binding site but binds to the protein at a site (Gla 24 and Gla21) which is also one of the Ca^{2+} binding sites.

Ultracentrifugation measurements of Pb^{2+} -osteocalcin

Previously published sedimentation equilibrium experiments demonstrated that oligomerization of osteocalcin from monomer to dimer is stimulated by the binding of Ca^{2+} [28,30]. Sedimentation equilibrium measurements conducted with Pb^{2+} -osteocalcin demonstrate comparable behavior. The weight average molecular weight of $8,070 \pm 198$ Da that is measured is appreciably greater than the calculated monomer molecular weight of 5,850 Da. As observed for Ca^{2+} -osteocalcin, the sedimentation profiles are best fit to a monomer-dimer association model [28,30]. The calculated dimerization constant of $3,493 \pm 630$ ($K_d = 286 \mu\text{M}$) indicates that 75% of the molecule exists as a dimer at the protein

concentration used in this experiment (1.70 mM). This association constant is only slightly weaker than that obtained in Ca^{2+} containing solutions [28,30].

All protein molecules have one conformation since there was only one set of NOE crosspeaks, no inconsistent NOE's and all NOE crosspeaks were used in the structure calculation with no distance constraint violations over 0.5 Å. This suggests that all observed NOEs were intramolecular rather than intermolecular at the dimer interface. A number of long range NOE interactions (Y42, Y46, and V22 etc.) were observed which could potentially be intermolecular NOES at a dimer interface rather than intramolecular NOES. To test this possibility we omitted these long range NOES from the structure calculation and obtained the same general backbone fold and same secondary structural characteristics with the exception of the close proximity of those hydrophobic side chains responsible for the observed NOEs. Also, identical 1D NMR spectra were obtained with the shifted resonances from Tyr and Val observed at both 50 µM protein (78 % monomer, 22 % dimer) and at 1.70 mM protein (25 % monomer, 75% dimer). The fact that the same hydrophobic interactions occur in the monomer suggests that they are intramolecular. A weight average molecular weight of $6,289 \pm 104$ was obtained for apo-osteocalcin using sedimentation equilibrium analysis. This indicates that the metal free protein is 90–95% monomeric under these experimental conditions. The differences between the weight average molecular weights of apo- and Pb^{2+} -osteocalcin suggest a metal mediated self-association. The data is highly suggestive of a symmetric dimer where the structure that was solved is of the individual monomers. Our data does not specify the location of the dimer interface but since it is a metal induced dimerization it may be at the Pb^{2+} binding site, similar to what was predicted with the NMR structure of Ca^{2+} -osteocalcin and observed in X-ray structures of porcine Ca^{2+} -osteocalcin and fish Mg^{2+} -osteocalcin [30,50]. The circulating concentration of osteocalcin in-vivo is in the nanomolar range indicating it is primarily monomeric. Osteocalcin is secreted from the osteoblast and accumulates on the bone with time, making an estimation of the concentration at the bone surface difficult. However it is most likely that after secretion from the osteoblast it binds to the bone and is in the monomeric form.

DISCUSSION

The genetic algorithm – molecular dynamics approach was used to predict the position of the lead binding site on osteocalcin. This method predicted one Pb^{2+} binding site at the carboxyl oxygens of Gla 24 and Gla 21 which is also one of the Ca^{2+} binding sites which we and others have previously observed.[28,49]. Both CD studies [29] and the 1D NMR here showed that one Pb^{2+} ion per osteocalcin molecule induced the maximum conformational change in the protein. In the CD and 1D NMR studies increased additions of lead did not alter the structure of the protein. Although lead ions have been shown to have high affinity for the SH groups of Cys residues, the two Cys residues in osteocalcin are involved in a disulfide bond. More importantly, Pb^{2+} has been demonstrated to bind to the same site as Ca^{2+} in osteocalcin since it can displace Ca^{2+} ions from the protein [51]. The chemical shifts of the Gla β and γ protons were shown to be altered similarly in the 1D spectrum as would be expected if the metal ion were bound to the same site. Since Ca^{2+} has been shown to bind to oxygen containing ligands in osteocalcin [28,30] we confined the search to O-O binding sites. There was no evidence of Pb^{2+} binding to residues other than Glas.

The lead binding site and coordinating oxygens of Gla 24 and Gla 21 were similar to one of the Ca^{2+} binding sites previously reported [28,30]. Lead, at a stoichiometry of only 1:1 can induce the same fold in the protein as calcium, at 3:1. We suggest that one Pb^{2+} ion binds tightly to the conformationally important binding site, while it takes the binding of 2 Ca^{2+} ions before the conformationally important site is occupied. The increased number of Ca^{2+} ions bound to the structure may be important in neutralizing the charge on the Gla residues, serving to stabilize the helical regions and may explain the decreased secondary structure seen in Pb^{2+} -osteocalcin. We can propose why Gla 24 and Gla 21 form the conformationally important binding site by examining the structure and the 2D NMR interactions. The tertiary structure and hydrophobic core are formed from long range hydrophobic interactions between the side chains of Y42 and Y46 with those of R20, V22, and C23 as shown in Figure 2 and 5C. The structure (Figure 5C and 6) shows that Pb^{2+} coordination between Gla24 and Gla21 promotes a helical turn in this region allowing R20, V22, and C23 to be in closer proximity to each other, facilitating the interaction of these side chains with those of Y42 and Y46. As shown by CD [29], the apo-protein is a random coil and does not exhibit the shifted aromatic and aliphatic side chain resonances in the 1D NMR spectrum (Figure 1b), indicating Ca^{2+} or Pb^{2+} are needed for this structural change. The D30 and Gla24 Ca^{2+} binding site may not be as important for the tertiary structure because the orientation of the Gla region to the D30-H35 helical region is due to the disulfide bond between C29–C23 which would be present even in the apo-protein. This Ca^{2+} binding site may serve to further stabilize the conformation. The Gla17 residue has been shown to be important for the secondary structure of the protein but not the tertiary structure. A 2 Gla form of osteocalcin has been reported to be observed especially in elderly humans where Gla17 is undercarboxylated and often exists as Glu17 (52). While this form of osteocalcin has been shown by CD to exhibit very little α -helical structure upon Ca^{2+} addition (53), there is evidence of tertiary structure. We have collected 1D and 2D NMR data on the Ca^{2+} complexed 2 Gla osteocalcin and we see similar long range hydrophobic interactions as are shown in Figure 2 (unpublished data). These observations lend support to the Gla24 and Gla21 binding site being the coordination site for Pb^{2+} and the important site for the formation of the hydrophobic core. There may be weaker binding sites for Pb^{2+} on the protein, as was seen for Ca^{2+} in the x-ray structure of porcine Ca^{2+} -osteocalcin [49], but there is one tight binding sight which can induce a similar fold to Ca^{2+} -osteocalcin.

Our study shows that Pb^{2+} , at a stoichiometry of 1:1, can induce a similar fold in a protein as Ca^{2+} but at a lower stoichiometry. The fact that the Pb^{2+} ion has a larger ionic radius than Ca^{2+} (1.2 Å and 0.99 Å respectively) may play a role in this. Other macromolecules have shown Pb^{2+} binding at a lower stoichiometry but the mechanism for this is not fully understood. Lead was found to bind principally to one of the two zinc binding sites of the ALAD enzyme and inhibit the enzyme but producing very little conformational change [54]. Lead binds with very high affinity to a thrombin binding aptamer (TBA), d(GGTGGTGTGGTTGG), inducing a folded quadruplex structure at a stoichiometry of only 1:1 (55) in contrast to the binding of 2 K^{+} cations normally needed for folding [56]. The ability of Pb^{2+} to bind tightly to macromolecules and induce similar conformational changes at a lower stoichiometry may be important in understanding its toxic effects.

We have previously shown that both Ca^{2+} and Pb^{2+} can increase the binding of osteocalcin to bone mineral hydroxyapatite as compared to the unstructured apo-protein (29). We proposed that, similar to Ca^{2+} at a stoichiometry of 3:1, Pb^{2+} may induce a more compact structure in osteocalcin allowing more molecules to fit on the crystal as compared to the apo-protein. In this paper we have shown that Pb^{2+} , at a stoichiometry of only 1:1, can induce a more compact structure in the protein supporting that hypothesis. Lead induces a more compact structure in osteocalcin with the C-terminus looping back and forming a hydrophobic core in the molecule as compared to the apo-protein which is an unstructured random coil. It is possible to illustrate the binding of Pb^{2+} -osteocalcin to bone mineral. It has been proposed that osteocalcin can bind to hydroxyapatite via the free uncoordinated oxygen ions of the Gla carboxyl groups binding to calcium in the hydroxyapatite lattice [57]. The structure of Pb^{2+} -osteocalcin in the mineral binding position is shown in Figure 6 where the free, uncoordinated Gla COOH groups are clearly labeled. The spacings between the free oxygen ions of the Gla carboxyl groups can be measured from the Pb^{2+} -coordinated structure of osteocalcin. In our study, the distances were measured from the best final structure and the errors were calculated from averaging these positions in all structures in the molecular dynamics simulation, taking into account the flexibility in the side chains of the Gla residues. The chi side chain angles are more constrained in Gla 24 and Gla 21 because the lead is coordinated between 2 residues whereas those in Gla 17 are more flexible since this residue is uncoordinated. The spacing between oxygen ions of Gla 24 and Gla21 is $8.61 - 9.57 \pm 1.00 \text{ \AA}$ and oxygen ions of Gla21 and Gla17 is $16.8 \pm 2.5 \text{ \AA}$. From reported x-ray data, the calculated spacings between calcium in hydroxyapatite are 3.44, 4.06, 5.45, 5.84, 6.33, 6.88 and $9.43 \text{ \AA}$ (57,58). The intercalcium distance of $9.43 \text{ \AA}$ correlates with the distance between the side chains of Gla 24 and Gla21, and Gla21 and Gla17 (a multiple of 9.4). The unit cell dimensions of the hydroxyapatite crystal are $a=b=9.43 \text{ \AA}$ and $c=6.88 \text{ \AA}$ (58). The intercalcium distance of $9.43 \text{ \AA}$ is located within the ac and ab faces of the unit cell (58). Figure 8 illustrates how the uncoordinated oxygens of the Gla carboxyl groups in the Pb^{2+} structure can interact with the calcium atoms in hydroxyapatite. This is similar to the proposed mineral binding of Ca^{2+} -osteocalcin [28] also shown in Figure 8B.

There is very little structural information on Pb^{2+} complexed proteins, especially at physiological pH values. From the structure proposed here and our previously published functional data for Pb^{2+} -osteocalcin [29], it is possible to propose one mechanism for the effect of Pb^{2+} at the molecular level for Ca^{2+} binding proteins. Our group and others have previously reported that at supraphysiological levels of Ca^{2+} (5–6 mM) in vitro, a conformational change in osteocalcin occurs which enhances its binding to hydroxyapatite [29,57]. This is due to the formation of a more compact tertiary structure, which allows a greater occupancy on the mineral surface. By comparison, we found that the addition of very low Pb^{2+} levels (1 μM), to physiological Ca^{2+} levels (1 mM) caused a significant 40% increase in the amount of hydroxyapatite mineral bound osteocalcin over that observed at 1 mM Ca^{2+} alone [29]. This is because metal free apo-osteocalcin does not bind as well to hydroxyapatite, whereas metal complexed osteocalcin shows greater binding [29]. At physiological Ca^{2+} levels of 1 mM and a K_d of 1.25 mM for Ca^{2+} -osteocalcin all of the protein is not bound to Ca^{2+} . Thus the added amount of Pb^{2+} produces a higher fraction of

metal bound protein than that which is produced by 1 mM Ca^{2+} alone. Since Pb^{2+} has a much higher affinity for the protein (K_d of 85 nM,) all of the 1 μM Pb^{2+} would be bound and 1 mM Ca^{2+} would complex a fraction of the remaining protein [29]. Since Pb^{2+} , at a stoichiometry of only 1:1, induces a similar, more compact fold in the protein and similar hydroxyapatite binding as Ca^{2+} , its added presence produces an increased effect on the hydroxyapatite binding [29]. Thus, conformational changes in Ca^{2+} -binding proteins, and subsequent molecular processes, which would normally occur only when extracellular calcium levels are increased, may also occur with lead exposure. This effect, produced by a foreign ion Pb^{2+} , could amplify normal Ca^{2+} regulated physiological processes in such proteins. Another Ca^{2+} binding protein, calmodulin, is effected similarly by lead. Lead was shown to bind to, induce a conformational change and activate calmodulin, similarly to Ca^{2+} , at very low levels [19]. The high affinity and/or lower stoichiometry binding of lead to proteins and subsequent disturbance of calcium regulated processes may contribute to a mechanism of lead toxicity in Ca^{2+} binding proteins.

Bone is the major reservoir of body lead. Low blood levels can cause a significant accumulation of Pb^{2+} in bone over time. Bone lead is a marker of past exposure with a half-life of 16 years [59] and has been found to increase with age [60]. Lead has been shown to displace Ca^{2+} in bone mineral hydroxyapatite [61]. Bone lead levels of 5–31 $\mu\text{g/g}$ bone have been reported which reflect lead values from nonexposed as well as exposed sections of the population [6,62,63]. In addition, there is evidence that bone extracellular fluid is compartmentalized, which is suggested by the existence of ionic gradients between bone extracellular fluid and systemic extracellular fluid [64,65]. Since bone is the major reservoir for lead, it is possible that Pb^{2+} concentrations could be increased around the bone surface or within the extracellular fluid and available for interaction with osteocalcin.

The binding of osteocalcin to bone mineral is regulated by calcium. Our in-vitro results presented here as well as our previous hydroxyapatite binding study with Pb^{2+} [29] suggest that lead may alter the distribution between bound and free osteocalcin in-vivo. Lead has been shown to increase bone resorption and decrease bone formation rates in animal studies resulting in a reduced bone density [11,13–15]. Recent evidence has suggested that osteocalcin is involved in the regulation of bone remodeling and it is possible that osteocalcin plays a role in the detrimental effects of lead on bone. Studies with the osteocalcin-depleted knockout mouse have shown that osteocalcin acts to limit bone formation [66]. Osteocalcin can also play a role in regulating the crystalline structure of bone as demonstrated by the fact that hydroxyapatite crystals are smaller and “less mature” in osteocalcin-depleted (knockout) mice compared to wild type [67]. Several earlier studies have also linked osteocalcin to bone resorption [68–70]. It is quite conceivable that elevated lead levels may affect normal bone turnover or change the mineral phase of bone as a consequence of changes in osteocalcin binding properties. This is currently being investigated with in-vivo studies.

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Figure 1.

A) ^1H 1D NMR apo-osteocalcin and at various Pb^{2+} /osteocalcin ratios. The shifted Tyr resonances are clearly marked. B) ^1H 1D NMR spectra of osteocalcin. The lower spectrum represents apo-osteocalcin, the middle spectrum represents Ca^{2+} /osteocalcin at a ratio of 3 and the top spectrum represents Pb^{2+} /osteocalcin at a ratio of 1. The signal at 4.6 ppm is from the H_2O protons. The protons from some shifted hydrophobic residues with both Ca^{2+} and Pb^{2+} are clearly marked. C) ^1H 1D NMR of osteocalcin at a Ca^{2+} /osteocalcin ratio of 1 (lower spectrum) and at a Pb^{2+} /osteocalcin ratio of 1 (upper spectrum).

Figure 2.

The two dimensional ¹H NMR NOESY spectra of Pb²⁺-osteocalcin. The aromatic-aliphatic proton region of the NOESY spectrum collected in H₂O at 37 °C with a 150 ms mixing time. Long range contacts involving aromatic side chain protons are labeled.

Figure 3.

Summary of NMR constraint data. The NOE constraints per residue are shown in (A) where black bars indicate long range NOES, dark gray bars indicate medium range NOES, light gray bars indicate sequential NOES and white bars indicate intrarresidue NOES. A summary of NMR restraints for Pb^{2+} -osteocalcin is shown in (B). The black circles indicate hydrogen bonds derived from multiple temperature experiments. The horizontal lines indicate backbone NOES with the thickness representing intensity. The H_α and $^{13}\text{C}_\alpha$ chemical shift deviations from random coil values are also shown.

Figure 4.

The structured region (residues 16-49) of the 10 best conformers of Pb^{2+} -osteocalcin. Superposition of the backbone atoms for residues 17-47 is shown in A, 21-47 is shown in B, 27-34 is shown in C, and 21-25 with the heavy side chains is shown in D. The RMSD's for the various regions are given in the Structural Statistics in Table 1.

Figure 5.

The ribbon diagrams of Pb^{2+} - and Ca^{2+} -osteocalcin. Pb^{2+} -osteocalcin is shown in Figure A at a ratio of $\text{Pb}^{2+}/\text{Ost} = 1$. The N-terminal region (residues 1–15) is unstructured and cannot be confined to a fixed orientation with respect to the rest of the molecule. There is a core in the C-terminal region (residues 16–49), which has helical content. The 3 Glu residues (X17, X21, X24) are surface exposed. The previously published NMR structure of Ca^{2+} -osteocalcin, at a ratio of $\text{Ca}^{2+}/\text{Ost} = 3$, is shown in Figure B for comparison (reprinted with permission from Dowd et al. *Biochemistry* 42: 7769–7779). Some of the hydrophobic side chains within the core of the Pb^{2+} -osteocalcin molecule are shown in Figure C. Long range interactions were observed between the aromatic and aliphatic side chains of F45 and L16 as well as Y46, Y42 and V22, C23 and C29.

Figure 6.

A) The lead coordinated structure of osteocalcin. The structured region of Pb^{2+} -osteocalcin (residues 16-49) after molecular dynamics simulation is shown in this figure where the lead ion is shown in yellow. A genetic algorithm determined the lead placements and the structures were then subjected to a molecular dynamics simulation as described in Materials and Methods. B) The lead bound structure in the bone binding orientation where the free, uncoordinated carboxyl oxygens on the Gla (X) residues are clearly labeled. The free oxygen ions of the Gla carboxyl groups are OE2 and OE1 of Gla 24, OE2 and OE3 of Gla 21 and OE1, OE2, OE3 and OE4 of Gla 17.

Figure 7.

A) A proposed mechanism for Pb^{2+} - osteocalcin interacting with bone mineral. The free carboxyl groups labeled in Figure 7 can bind to calcium ions in the mineral. The accessible C terminus in the bone binding orientation is labeled. B) A proposed mechanism for Ca^{2+} - osteocalcin interacting with bone mineral. (Reprinted with permission from Dowd et al. *Biochemistry* 42: 7769–7779).

Table 1

Structural Statistics

Total Number of NOE Constraints	552
Intraresidue	65
Sequential	230
Medium range	169
Long Range	88
Largest distance constraint violation (Å)	0.30
RMSD	
Backbone (Å)	1.70 (residues 21-47)
	0.57 (residues 27-34)
Heavy Atoms (Å)	
	2.60 (residues 21-25)
Deviations from idealized geometry	
Bonds (Å)	0.00343
Angles (deg)	0.4473
van der Waals energy (kcal/mol)	-206.6
Ramachandran Plot	45 % most favorable region
	55 % in additionally and generously allowed