



ELSEVIER

Biochimica et Biophysica Acta 1535 (2001) 153–163



www.elsevier.com/locate/bba

The effect of Pb^{2+} on the structure and hydroxyapatite binding properties of osteocalcin

T.L. Dowd ^{a,*}, J.F. Rosen ^a, L. Mints ^b, C.M. Gundberg ^c

^a Department of Pediatrics, Montefiore Medical Center, Albert Einstein College of Medicine, Moses Bldg. Room 401, 111 East 210th Street, Bronx, NY 10467, USA

^b Department of Biochemistry and the Laboratory for Macromolecular Analysis and Proteomics, Albert Einstein College of Medicine, Bronx, NY, USA

^c Department of Orthopedics, Yale University School of Medicine, New Haven, CT, USA

Received 14 June 2000; received in revised form 2 October 2000; accepted 12 October 2000

Abstract

Lead toxicity is a major environmental health problem in the United States. Bone is the major reservoir for body lead. Although lead has been shown to impair bone metabolism in animals and at the cellular level, the effect of Pb^{2+} at the molecular level is largely unknown. We have used circular dichroism (CD), and a hydroxyapatite binding assay to investigate the effect of Pb^{2+} on the structure and mineral binding properties of osteocalcin, a noncollagenous bone protein. The CD data indicate Pb^{2+} induces a similar structure in osteocalcin as Ca^{2+} but at 2 orders of magnitude lower concentration. These results were explained by the more than 4 orders of magnitude tighter binding of Pb^{2+} to osteocalcin ($K_d = 0.085 \mu\text{M}$) than Ca^{2+} ($K_d = 1.25 \text{ mM}$). The hydroxyapatite binding assays show that Pb^{2+} causes an increased adsorption to hydroxyapatite, similar to Ca^{2+} , but at 2–3 orders of magnitude lower concentration. Low Pb^{2+} levels (1 μM) in addition to physiological Ca^{2+} levels (1 mM) caused a significant (40%) increase in the amount of mineral bound osteocalcin as compared to 1 mM Ca^{2+} alone. These results suggest a molecular mechanism of Pb^{2+} toxicity where low Pb^{2+} levels can inappropriately perturb Ca^{2+} regulated processes. In-vivo, the increased mineral bound osteocalcin could play a role in the observed low bone formation rates and decreased bone density observed in Pb^{2+} -intoxicated animals. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Osteocalcin; Lead; Calcium; Circular dichroism; Hydroxyapatite

1. Introduction

Lead toxicity is a major public health problem in the United States. Despite governmental regulations, elevated blood lead levels ($\geq 0.5 \mu\text{M}$) have been re-

cently reported to be highest among very young children (aged 1–5 years) as well as older adults ≥ 50 years old [1].

Bone is the major reservoir of body lead accounting for 75% of total body lead in normal children and over 90% in adults [2,3]. Bone lead values have been found to be elevated in lead-intoxicated children [4] and in adults who have either been occupationally exposed or have had past exposure to higher environmental levels [5,6]. Bone is known to be an

* Corresponding author. Fax: +1-718-920-4377;
E-mail: dowd@acom.yu.edu

important endogenous source capable of releasing stored lead to other soft tissues and organs in normal and pathological states.

Lead has been found to have detrimental effects on bone. Chronic low level lead exposure has been associated with decreased stature and chest circumference in children less than 7 years of age [7,8]. Several studies have indicated that lead can effect both bone formation and resorption [9–11] resulting in reduced bone density in experimental animals [12]. At the cellular level, lead has been shown to perturb calcium homeostasis and alter cellular metabolism or activity of osteoclasts [13] and osteoblasts [14–19]. Little is known about the effect of lead at the protein level. The comparison of the effect of a toxic metal ion, Pb^{2+} , with an essential metal ion, Ca^{2+} , on the structure and activity of a bone matrix protein from a target tissue and from a site of concentrated storage has not been reported.

Osteocalcin (49 amino acids) is an abundant non-collagenous matrix protein found in bone [20,21] and dentin [22]. It is synthesized exclusively by osteoblasts [23,24] and odontoblasts [25]. Osteocalcin is deposited in the mineralized matrix, but a small amount is detected in serum where it circulates at levels that reflect the metabolic state of bone in normal and pathologic conditions [26]. Osteocalcin contains three γ -carboxyglutamic acid residues (Gla) which can bind Ca^{2+} in solution and in the mineral phase [27]. Several early in vitro studies have suggested a role for osteocalcin in bone resorption [28,29], osteoclast differentiation [29–31] or crystal formation and growth [32–34]. However, recent studies indicate that osteocalcin may play a regulatory role in bone formation and remodeling. In genetically engineered osteocalcin-depleted (knockout) mice, there was increased bone mass and consequently increased biomechanical strength while bone formation rates were increased [35]. Subsequent studies indicated an immature mineral phase in these animals [36].

We have previously shown that low levels of Pb^{2+} can displace Ca^{2+} from osteocalcin and lead was shown to have a much higher affinity for osteocalcin than that of Ca^{2+} [37] at very low ionic strength. Circular dichroism studies have shown Ca^{2+} induces an α -helical conformation in osteocalcin which correlates with maximum adsorption of osteocalcin to

hydroxyapatite [38]. Another essential metal, Mg^{2+} , was also found to induce an α -helical conformation yet inhibit the binding of osteocalcin to hydroxyapatite [39]. The effect of a toxic metal, Pb^{2+} , on the secondary structure and hydroxyapatite binding properties of osteocalcin have not been determined. Lead may exert its toxic effect by altering the secondary structure of osteocalcin and decreasing its adsorption to hydroxyapatite. Alternatively, Pb^{2+} may induce a similar secondary structure in the protein as Ca^{2+} and induce an inappropriate increase in hydroxyapatite binding at much lower concentrations.

The purpose of this paper is to investigate the effect of Pb^{2+} on the structure and functional properties of osteocalcin using circular dichroism spectroscopy and hydroxyapatite binding assays. Similar data were collected for the essential metal, Ca^{2+} , and apo-osteocalcin for comparative purposes.

2. Experimental procedures

2.1. Preparation of osteocalcin

Bovine osteocalcin was prepared from calf bone by a previously described method [40]. The purity of the protein was determined by polyacrylamide gel electrophoresis, reverse-phase high-performance liquid chromatography (HPLC) and N-terminal amino acid analysis. This protein was used for the CD studies and hydroxyapatite binding assays.

Some of the protein in this study was also synthesized using solid phase peptide synthesis methodology on an ABI 433 peptide synthesizer using Fmoc protocols [41]. Reagents (piperidine, HOBT/HBTU activation kit, diisopropylethylamine, NMP, DCM and Fmoc standard amino acids) were purchased from ABI. Fmoc γ -carboxyGlu(OtBu)₂-OH (Gla) was purchased from Bachem. Fmoc preloaded amino acid Wang resin was purchased from Advanced Chem Tech.

Peptide resins were cleaved/deprotected by a mixture of TFA/thioanisole/EDT/phenol/H₂O for 2.5 h, precipitated by tert-butyl methyl ether and then washed. The disulfide bond between the two Cys residues was formed by slow air oxidation methods [42] and the protein was purified by reverse-phase HPLC. Synthetic bovine osteocalcin was character-

ized by analytical HPLC, electrospray mass spectrometry and amino acid analysis.

The synthetic protein was found to give similar CD and one-dimensional (1D) NMR spectra and to bind to hydroxyapatite in a similar manner as the osteocalcin prepared from calf bone. The synthetic protein was used for some of the CD experiments in this paper.

To ensure that there was no residual metal binding to osteocalcin resulting from the various preparation procedures, low concentrations of osteocalcin ($\sim 200 \mu\text{M}$) were treated with 2 mM EGTA. The protein was then concentrated in a Centricon 3 concentrator and subsequently washed five times (1/10 dilution) with distilled deionized H_2O to decrease the concentration of EGTA. The final protein concentrate contained negligible levels of EGTA which were then further diluted by a factor of 1000 for protein concentrations needed in this study.

2.2. Circular dichroism experiments

Bovine osteocalcin solutions were prepared at protein concentrations at or near $50 \mu\text{M}$ and dissolved in 150 mM NaCl and 20 mM Tris at a pH of 7.4. Protein concentrations were measured spectrophotometrically using the absorbance coefficient $A_{1\text{cm}}^{1\%} = 13.3$ at 280 nm.

CD spectra were collected at various concentrations of Ca^{2+} and Pb^{2+} (see Section 3) at 37°C .

All CD spectra were collected on a Jasco J-720 spectropolarimeter using a 0.05 cm pathlength water-jacketed cuvette regulated for temperature control at 37°C . Spectra were typically measured at 200–250 nm, using a bandwidth of 1.0 nm, a resolution of 0.5 nm and spectra were the result of the average of three accumulations. Each spectrum was corrected for solvent effects. Secondary structure estimates were calculated from the CD spectra using the Yang method of analysis [43].

2.3. Determination of the K_d for Pb^{2+} -osteocalcin

For the determination of the K_d for Pb-osteocalcin, a $0.25\text{--}0.5 \mu\text{M}$ protein solution (dissolved in the buffer described above) was titrated with lead nitrate and CD spectra were collected at 37°C in a 5 cm pathlength water-jacketed cuvette. The total Pb^{2+}

concentrations added to all solutions were determined by graphite atomic absorption spectroscopy using a modification of a previously published method [44]. The molecular ellipticity at 222 nm was recorded for the metal-free protein (apoprotein) as well as at each addition of Pb^{2+} . The change in the molar ellipticity at 222 nm from that of the apoprotein ($\Delta\theta$) was calculated for each addition of Pb^{2+} and plotted as function of total Pb^{2+} added. The data were fit to the following equation [45]:

$$\Delta\theta = \Delta\theta_{\text{max}}[\text{Pb}^{2+}]/K_d + [\text{Pb}^{2+}] \quad (1)$$

where $\Delta\theta$ is the change in the molar ellipticity at 222 nm between the protein with added Pb^{2+} and that of the apo-protein, $[\text{Pb}^{2+}]$ is the free Pb^{2+} concentration, $\Delta\theta_{\text{max}}$ is the maximum change in $\Delta\theta$ and K_d is the dissociation constant for the Pb^{2+} -osteocalcin complex. The value for free Pb^{2+} , $[\text{Pb}^{2+}]$, at each addition of total Pb, $[\text{Pb}_T]$, was calculated from the difference between the measured $[\text{Pb}_T]$ and the concentration of Pb bound, $[\text{Pb}_B]$, calculated from the following equation [37]:

$$[\text{Pb}_B] = (K_d + [\text{Ost}_T] + [\text{Pb}_T] - ((K_d + [\text{Ost}_T] + [\text{Pb}_T])^2 - 4[\text{Ost}_T][\text{Pb}_T])^{1/2})/2 \quad (2)$$

where $[\text{Pb}_T]$, as measured from atomic absorption and the protein concentration $[\text{Ost}_T]$, were used to extract the parameters K_d and $\Delta\theta_{\text{max}}$ using Eqs. 1 and 2. The best values of K_d and $\Delta\theta_{\text{max}}$, giving the smallest difference between the observed and calculated $\Delta\theta$, were obtained iteratively minimizing the error square sum to obtain the best fit.

2.4. Hydroxyapatite binding assays

A stock solution of hydroxyapatite (Ca/P molar ratio of 1.67 ± 0.02 and surface area of $59.56 \text{ m}^2/\text{g}$ by N_2 adsorption) (5 mg/ml) was dissolved in 150 mM NaCl, 20 mM Tris (pH 7.4), and serially diluted to make hydroxyapatite solutions with final concentrations of 1.66, 0.833, 0.416, 0.21, 0.104, and 0.052 mg/ml. Hydroxyapatite in the serially diluted samples was quantitated by dissolving the crystal in 0.5 N HCl and quantitating total calcium with flame atomic adsorption spectroscopy. The quantitated hydroxyapatite correlated very closely ($r=0.994$) with hydroxyapatite concentrations calculated by serial

dilution. Unlabeled bovine osteocalcin ($\sim 25 \mu\text{g/ml}$), in 150 mM NaCl, 20 mM Tris (pH 7.4) with Ca^{2+} concentrations of 0, 1 or 6 mM or Pb^{2+} concentrations of 1 or 10 μM were added to the various hydroxyapatite suspensions and shaken vigorously for 1 h. The extent of crystal dissolution after vortex shaking was assessed by measuring total Ca^{2+} in the supernatant of the 1.66 mg/ml hydroxyapatite sample using flame atomic absorption spectroscopy. It was found that crystal dissolution was only 0.7% in these experiments and not affected by the presence of any metal ion. Nonspecific binding of osteocalcin was assessed for each concentration of metal ion. The suspensions were then centrifuged for 10 min at $10\,000\times g$ and an aliquot of the supernatant was quantitated by radioimmunoassay as previously described [40]. The total protein concentration available for binding to hydroxyapatite (1.4–2.4 μM), and the concentration of free, unbound osteocalcin in the supernatant from the various metal ion and hydroxyapatite containing solutions were determined. Briefly, the data was analyzed according to previously published methods and fit to a Langmuir isotherm model adapted to analyze the adsorption of proteins to apatite surfaces [39]:

$$C/Q = C/N + 1/NK$$

where C is the solution concentration of free osteocalcin in equilibrium with hydroxyapatite, Q is the amount of osteocalcin adsorbed per unit of hydroxyapatite surface (using the hydroxyapatite-specific surface area of $59.56 \text{ m}^2/\text{g}$), N (nmol/m^2) is the maximum number of adsorption sites per square meter of hydroxyapatite and K (ml/nmol) is the affinity of osteocalcin for the adsorption sites. Three or four experiments were conducted on each Ca^{2+} (0, 1 and 6 mM), Pb^{2+} (10 μM) and Ca^{2+} (1 mM) plus Pb^{2+} (1 μM) containing solution and all were performed in duplicate or triplicate. Plots of C/Q vs. C were constructed for all free protein concentrations in a particular solution (30–50 points) and linear regression was performed on all the data points. The 95% confidence limits were also calculated for the slope and intercept using these plots. In addition, the area of a single binding site, $(1/NM)$, was calculated for osteocalcin in each metal containing solution where the value of N is defined above and M is Avogadro's number.

3. Results

3.1. Circular dichroism studies

In order to assess and compare the effect of Pb^{2+} and Ca^{2+} on the gross features of the structure of osteocalcin, circular dichroic spectra were collected at various total Pb^{2+} and Ca^{2+} concentrations. Fig. 1A shows CD spectra at Ca^{2+} concentrations ranging from 0.1–6 mM. The CD spectrum of the apo-protein exhibits a negative band around 204 nm consistent with a random coil structure and very little signal at 222 nm. This is consistent with an α -helical content of $\leq 5\%$. A shift in the negative band to 208 nm, as well as an increasingly negative band observed at 222 nm, was observed with Ca^{2+} addition. This indicated that Ca^{2+} addition induces an α -helical conformation in the protein with the maximal conformational change occurring at 5–6 mM Ca^{2+} . Results from three different experiments were consistent with a Ca^{2+} -induced average α -helical content of 18%.

Fig. 1B shows a CD titration with Pb^{2+} . As with Ca^{2+} , increasing Pb^{2+} concentration induced a shift in the negative band from 202 (random coil) to 208 nm and the observation of a negative band at 222 nm. The average result from three different experiments indicated a Pb^{2+} -induced conformational change of 20% α -helix. In contrast to Ca^{2+} , the maximal conformational change occurred at Pb^{2+} concentrations which are 2 orders of magnitude lower than those required for a Ca^{2+} -induced conformational change (55 μM). The protein concentration in these experiments was 50 μM and the maximum change occurred at 55 μM Pb^{2+} , indicating very tight binding of Pb^{2+} to osteocalcin. The CD spectra in the presence of Ca^{2+} and Pb^{2+} were collected in the concentration range of 0.029–0.29 mg/ml osteocalcin with similar conformational changes observed at all protein concentrations. This suggests that the CD spectrum in this wavelength region is associated with a change in the backbone conformation of osteocalcin rather than due to protein-protein interactions.

3.2. K_d determination

We had previously reported that Pb^{2+} had a much

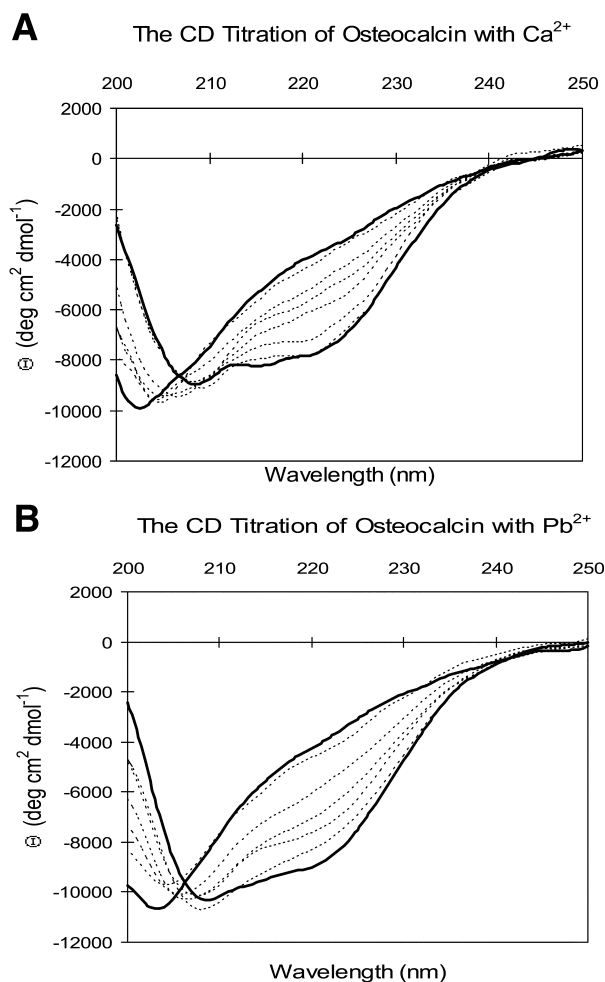
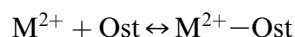


Fig. 1. (A) The effect of Ca²⁺ on the CD spectrum of osteocalcin, showing the CD spectrum for apo-osteocalcin (solid line, signal at 204 nm) and the effect of increasing amounts of Ca²⁺ (0.1, 0.5, 0.7, 1.0, 3.0, 5.0 mM, dotted lines) and 6.0 mM Ca²⁺ (solid line, signal at 208 and 222 nm) on the spectrum. An increasing shift of the signal from 204 nm for apo-osteocalcin (random coil) to 208 and 222 nm (α -helical conformation) with increasing Ca²⁺ is observed. (B) The effect of Pb²⁺ on the CD spectrum of osteocalcin, showing the CD spectrum for apo-osteocalcin (solid line, signal at 204 nm) and the effect of increasing amounts of Pb²⁺ (10, 25, 31, 40, 48 μ M, dotted lines) and 55 μ M Pb²⁺ (solid line, signal at 208 and 222 nm) on the spectrum. An increasing shift of the signal from 204 nm for apo-osteocalcin (random coil) to 208 and 222 nm (α -helical conformation) with increasing Pb²⁺ is observed.

lower K_d (higher affinity) for osteocalcin as compared to Ca²⁺ at low ionic strength ([NaCl] = 0) [37]. To ensure that this difference in affinity was maintained at physiological ionic strength (150 mM NaCl) K_d measurements were determined from CD

titration data. The calculations are described in the experimental section. Fig. 2A and B show the change in molar ellipticity relative to the metal-free protein (apoprotein) as a function of total Pb²⁺ and Ca²⁺, respectively. These data could be fit to a model of chemical equilibria with one binding site using Eqs. 1 and 2:



The best fit yielded a K_d for Pb²⁺-osteocalcin of 85 ± 12 nM and a K_d for Ca²⁺-osteocalcin of 1.25 ± 0.19 mM. These data show that Pb²⁺ binds

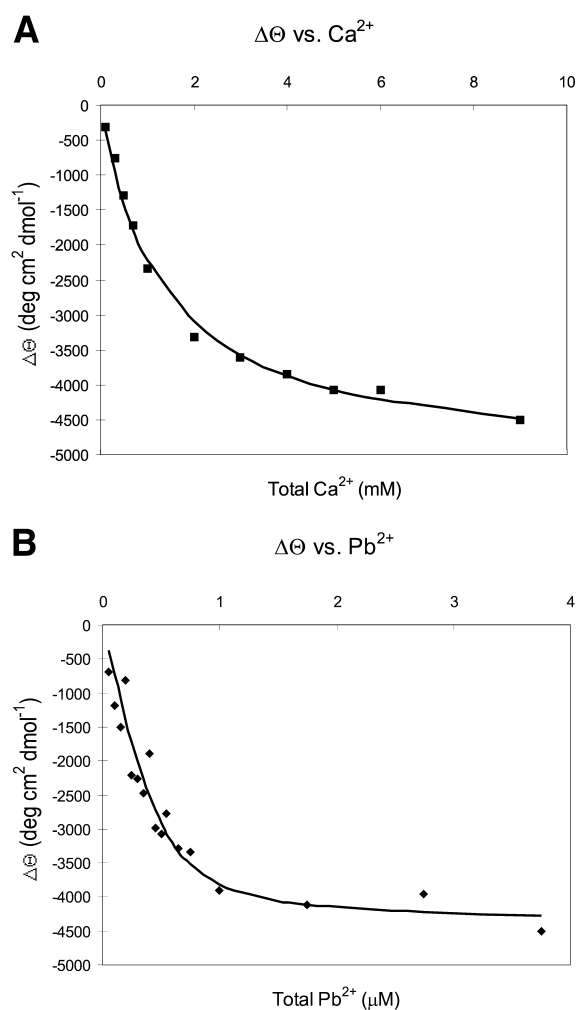


Fig. 2. (A) The change in molar ellipticity ($\Delta\Theta$) at 222 nm, with respect to the apo-protein, for osteocalcin with added Ca²⁺. The data could be fit to a one-binding-site model with a K_d of 1.25 ± 0.19 mM. (B) The change in molar ellipticity ($\Delta\Theta$) at 222 nm, with respect to the apo-protein, for osteocalcin with added Pb²⁺. The data could be fit to a one-binding-site model with a K_d of 0.085 ± 0.012 μ M.

to osteocalcin with an affinity which is over 4 orders of magnitude tighter than that of Ca^{2+} at physiological ionic strength. The data in this experiment could be fit to one binding site but does not rule out the existence of weaker binding sites. The purpose of the measurement was to explain the difference in concentration of the two ions required to induce the conformational changes. Weaker binding sites probably do not contribute to the observed conformational changes.

3.3. Hydroxyapatite binding assays

A comparison between the effect of Ca^{2+} and Pb^{2+} on the function of osteocalcin was assessed using hydroxyapatite binding assays. The data was analyzed as described in Section 2. The values for K and N for the apo-protein and at each Ca^{2+} and Pb^{2+} solution are reported in Table 1. For each solution, all data points (30–50) were used to calculate the best fit line as well as the 95% confidence limits on the line (errors in Table 1). For presentation purposes, the average values of C and C/Q (5–20 points) were calculated at each hydroxyapatite concentration and are shown in Fig. 3A–C for the different solution conditions.

Fig. 3A shows the concentration dependent effect of Ca^{2+} on the binding of osteocalcin to hydroxyapatite and Table 1 gives the binding parameters. Fig. 3A indicates an increase in the occupancy of the sites on the crystal with increasing total $[\text{Ca}]_{\text{T}}$ concentrations. There was a significant difference ($p \leq 0.05$) between the slopes of apo-osteocalcin (metal-free) and that induced by 1 mM $[\text{Ca}]_{\text{T}}$, indicating an increase of 30% in the occupancy of the sites on the crystal at physiological $[\text{Ca}^{2+}]_{\text{f}}$. The slope with 1 mM $[\text{Ca}]_{\text{T}}$ was significantly ($p \leq 0.05$) higher than that

with 6 mM $[\text{Ca}]_{\text{T}}$, indicating an increase of 25% in the occupancy of sites on the hydroxyapatite crystal with an increase in Ca^{2+} above the physiological level. The slope for the apo-protein (metal-free) was also significantly larger ($p \leq 0.05$) than that induced by 6 mM $[\text{Ca}]_{\text{T}}$. This indicated that addition of 6 mM $[\text{Ca}]_{\text{T}}$ results in a 62% increase in the number of adsorption sites on the hydroxyapatite crystal as compared to the metal-free apo-protein. These results can be explained by the fact that a greater fraction of the protein is complexed to Ca^{2+} at higher Ca^{2+} concentrations (44% at 1 mM and 83% at 6 mM). It was also observed that the maximum area of a single binding site decreased in the presence of Ca^{2+} . This is probably due to the Ca^{2+} -induced conformational change in the protein. Although the average values for the affinities (K) of osteocalcin for the sites on the crystal were larger in the presence of Ca^{2+} , they are not significantly different from the affinity for the metal-free apo-protein.

Fig. 3B shows a comparison between the metal-free apo-protein and the effect of saturating levels of Pb^{2+} (10 μM) on osteocalcin binding to hydroxyapatite. This figure shows that Pb^{2+} induced a similar effect on the binding of osteocalcin to hydroxyapatite as Ca^{2+} . However, due to the much higher affinity of Pb^{2+} for osteocalcin as compared to Ca^{2+} , a much lower metal ion concentration was necessary to induce the same amount of binding to hydroxyapatite. The difference in slopes between Ca^{2+} - and Pb^{2+} -treated osteocalcin and the apo-protein are clearly visible. The Pb^{2+} result is also due to the fact that at 10 μM Pb^{2+} most of the protein is complexed to Pb^{2+} ($\sim 95\%$) and in a conformationally altered state. There was a significant difference ($p \leq 0.05$) in the slopes between the metal-free apo-protein and that of 10 μM Pb^{2+} which indicated an

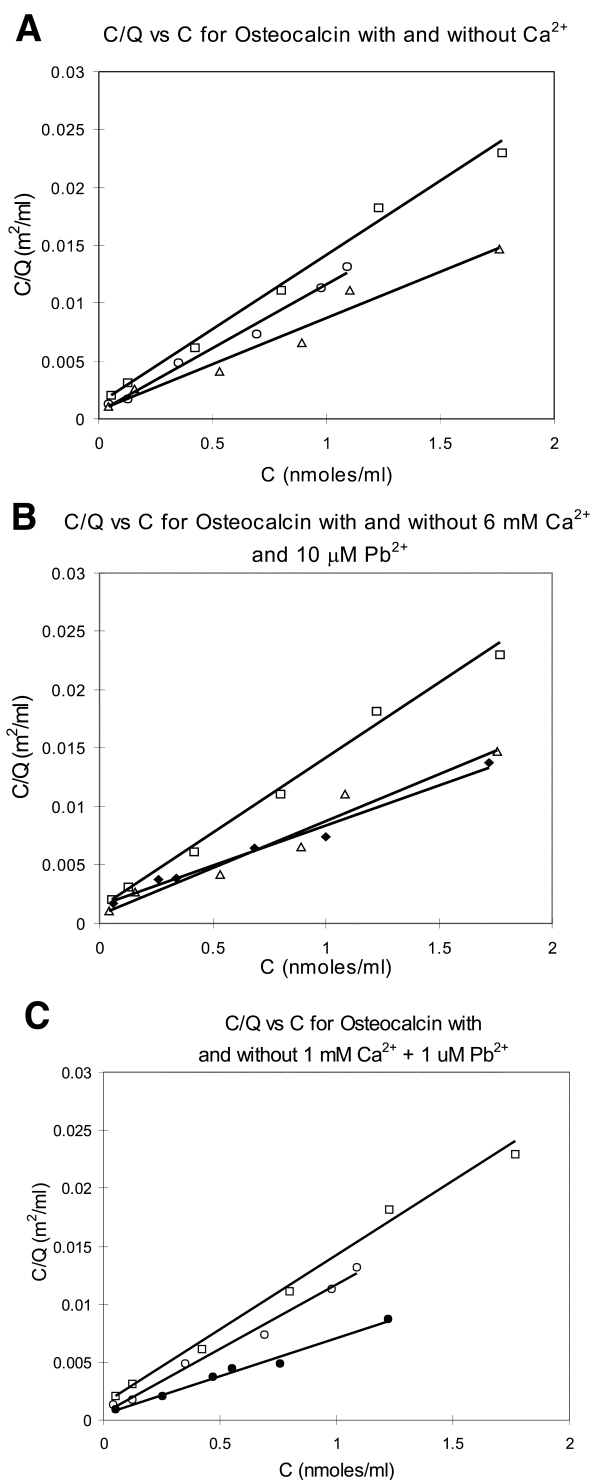
Table 1
Binding parameters for Apo-, Ca^{2+} - and Pb^{2+} -osteocalcin

	Apo-osteocalcin	1 mM Ca_{T}	6 mM Ca_{T}	10 μM Pb_{T}	1 mM Ca^{2+} +1 μM $\text{Pb}_{\text{T}}^{\text{a}}$
No. of binding sites (nmol/m ²)	77 ± 7	100 ± 9 ^b	125 ± 13 ^{b,c}	143 ± 22 ^{b,c}	140 ± 22 ^{b,c}
Binding constant, K (ml/nmol)	9.2 ± 4	12.5 ± 6	11.4 ± 5	7.0 ± 4	13 ± 5
Area of a single binding site (Å ²)	2076		1350	1195	

^aTotal protein concentration was 1.4–1.79 μM .

^bSignificantly different ($p \leq 0.05$) from apo-osteocalcin.

^cSignificantly different ($p \leq 0.05$) from 1 mM Ca^{2+} .



increase of 85% in the occupancy of sites on the crystal with 10 μM Pb²⁺. No significant difference was observed between the affinity constants with Pb²⁺ as compared to the metal-free apo-protein or

Fig. 3. (A–C) Binding isotherms for apo-osteocalcin in the presence of Ca²⁺ and Pb²⁺. Each point represents an average (5–25 points) at a particular hydroxyapatite concentration. The effect of Ca²⁺ (A) on the binding of osteocalcin to hydroxyapatite for apo-osteocalcin (□), osteocalcin with 1 mM Ca²⁺ (○), and 6 mM Ca²⁺ (△). The effect of Pb²⁺ (B) on the binding of osteocalcin to hydroxyapatite for apo-osteocalcin (□), osteocalcin with 6 mM Ca²⁺ (△), and 10 μM Pb²⁺ (◆). The binding isotherm for apo-osteocalcin (□), 1 mM Ca²⁺ (○) and 1 mM Ca²⁺ and 1 μM Pb²⁺ (●) is shown in C.

Ca²⁺ bound protein. As was seen with Ca²⁺, the area of a single binding site was also decreased, as compared to the apo-protein, but with much lower levels of Pb²⁺. This was most likely due to the conformational change induced by Pb²⁺ on the protein.

To investigate the effect of Pb²⁺ on the binding of osteocalcin to hydroxyapatite under physiologically relevant conditions the experiment was conducted in the presence of both 1 mM Ca²⁺ and 1 μM Pb²⁺. Fig. 3C shows a significant (40%) increase in the occupancy of the protein on the crystal from physiological Ca²⁺ levels with the added presence of 1 μM Pb²⁺. This effect is due to the added amount of Pb²⁺ producing a higher fraction of metal bound protein than that which is produced by 1 mM Ca²⁺ alone. Since Pb²⁺ has a much higher affinity for the protein, all of the 1 μM Pb²⁺ would be bound and 1 mM Ca²⁺ would complex 44% of the remaining protein. This would indicate that 75–85% of the protein would be in a metal complexed state at the total protein concentrations used in this experiment. Since Pb²⁺ induces a similar secondary structure and hydroxyapatite binding as Ca²⁺, its added presence produces an increased effect on the hydroxyapatite binding. The number of binding sites on the crystal in the presence of 1 mM Ca²⁺ and 1 μM Pb²⁺ was not significantly different from the number of binding sites induced by 6 mM Ca²⁺ or by 10 μM Pb²⁺ alone.

To ensure that the protein was not denatured and that it maintained its conformation during the mixing period with hydroxyapatite, CD spectra were collected after 1 h of vortex shaking on protein samples of the apo-protein, protein in 6 mM Ca²⁺ and 10 μM Pb²⁺. The CD spectra revealed that the protein conformation was indistinguishable from that obtained without shaking.

4. Discussion

Very little is known about the effect of a toxic metal on the secondary structure and functional properties of proteins. The effect of a toxic metal, Pb^{2+} , on the secondary structure and hydroxyapatite binding properties of osteocalcin has not been previously reported. Our results concerning the effect of Ca^{2+} on the structure and activity of osteocalcin was obtained for comparative purposes with a toxic metal. These results are in reasonable agreement with literature values reported previously for the effect of Ca^{2+} on osteocalcin. We found that Ca^{2+} induces an α -helical structure in 18% of the protein. This is in reasonable agreement with previous studies where values of 15–31% α -helix were observed by CD [46,47] for bovine Ca^{2+} -osteocalcin. The K_d for Ca^{2+} -osteocalcin measured in this study (1.25 mM) is also in reasonable agreement with previously reported K_d measurements, at physiological ionic strength, ranging from 0.8 mM to 3 mM depending on the species [48,49].

In this study we have shown that Pb^{2+} induces a similar percentage of α -helical conformation in osteocalcin as Ca^{2+} but at much lower concentrations. This can be explained by the over 4 orders of magnitude tighter binding of Pb^{2+} to osteocalcin as compared to Ca^{2+} . Previous studies have shown that the γ -carboxyglutamic acid residues (Gla) were necessary for binding Ca^{2+} in solution [27] and for the maximum α -helical conformational change detected by CD [38,47]. Our previous data [37] together with the CD data reported here suggest that Pb^{2+} interacts similarly with the Gla residues as Ca^{2+} . Using ^{43}Ca NMR and monitoring the ^{43}Ca T_1 we showed that Pb^{2+} can displace Ca^{2+} from osteocalcin [37]. Since Pb^{2+} can displace Ca^{2+} it must bind to similar coordinating ligands. The ^1H NMR spectra also showed similar alterations in resonances where $\text{Gla}\beta$ and $\text{Gla}\gamma$ protons occur with Ca^{2+} or Pb^{2+} addition [37]. The data suggest that Pb^{2+} and Ca^{2+} bind to the same site where at least some of the coordinating ligands are the same for both metal ions and that the Gla residues are involved in coordinating both Ca^{2+} and Pb^{2+} .

We found no significant difference (within our experimental error) in the binding affinities, K , of the protein for the crystal at any of the ion concentra-

tions studied. An earlier study reported that Ca^{2+} (5 mM) induced an α -helical conformation in osteocalcin and enhanced the binding of osteocalcin to hydroxyapatite [38,39]. The previous study, using ^{125}I -labeled chicken osteocalcin, found that the enhanced binding with Ca^{2+} was due to the affinity of Ca^{2+} -osteocalcin to hydroxyapatite being greater than that of the apo-protein and that the number of binding sites was the same [39]. Our results for Ca^{2+} may differ on the binding parameters because of the species difference and the fact that ^{125}I -labeled protein was used for estimation of binding in the previous study rather than by direct measurement as we have used here.

Our results on the effect of Pb^{2+} on osteocalcin binding to hydroxyapatite have not been previously reported. Both Ca^{2+} and Pb^{2+} are shown to increase the number of sites occupied on the crystal by the protein. Pb^{2+} was shown to increase the number of sites on the crystal at 2–3 orders of magnitude lower concentration than Ca^{2+} . These results are explained by the increased amount of protein complexed to the metal ions and in a conformationally altered state at higher free metal ion concentrations. The greater affinity of osteocalcin for Pb^{2+} as compared to Ca^{2+} explains the concentration difference required for the same amount of binding. In our study, the fact that the binding affinity for the crystal did not change with Ca^{2+} or Pb^{2+} addition suggests that similar functional groups on the amino acids and at the adsorption sites are involved in the adsorption process. The area of a single binding site was found to decrease with concentrations of Ca^{2+} and Pb^{2+} where most of the protein is in the metal bound state (1350, 1195 and 2076 \AA^2 for Ca^{2+} -, Pb^{2+} - and apo-osteocalcin, respectively). This suggests that the apo-protein may have a more elongated conformation than that of the more structured Ca^{2+} and Pb^{2+} protein complexes. A 2D NMR study on rabbit Ca^{2+} -osteocalcin detected a set of long range NOEs indicating the proximity of Leu2, Leu36, Ala33 and Phe45 as well as Val36 to Tyr42 which served to define a hydrophobic core for the molecule [46]. We have previously shown for bovine osteocalcin that Pb^{2+} induces a similar 1D NMR spectrum as Ca^{2+} with similar alterations in hydrophobic residues as reported in rabbit Ca^{2+} -osteocalcin [37]. The alterations in the resonances of hydrophobic residues

with Ca^{2+} and Pb^{2+} in bovine osteocalcin were not observed in the 1D NMR spectrum of the metal-free apo-protein. This suggests that a hydrophobic core may be formed with Ca^{2+} and Pb^{2+} in bovine osteocalcin as well. The α -helical structural change observed by CD and the hydrophobic interactions between nonsequential residues may produce a less elongated structure in Ca^{2+} - and Pb^{2+} -osteocalcin, as compared to the apo-protein, and may allow more molecules to fit on the crystal. It is also possible that the metal induced structural change produces a conformation in the protein which is more compatible with the binding sites on the crystal. A 2D NMR high resolution structure of bovine Ca^{2+} - and Pb^{2+} -osteocalcin is currently in progress which will be able to directly address this.

A model for Ca^{2+} -osteocalcin was proposed [38] in which Ca^{2+} induces an α -helical structure whereby the spacing between the Gla residue side chains coincides with the spacing between the Ca^{2+} atoms in the hydroxyapatite crystal [50]. This allows for binding to the bone via uncoordinated Gla residues. The conformational change observed with Ca^{2+} and Pb^{2+} in our study is also consistent with a structure which is more compatible than the apo-protein with the binding sites on the crystal. Although it has not yet been shown, it is quite conceivable that the interaction of Pb^{2+} -osteocalcin with hydroxyapatite is similar to the proposed model for Ca^{2+} -osteocalcin. In this study lead was shown to induce a similar structure in the protein. It has been reported that lead can substitute for Ca^{2+} in the hydroxyapatite crystal [51] so that the spacing between the Pb^{2+} atoms would be the same as that between the Ca^{2+} atoms. In vitro studies have shown that Pb^{2+} can displace Ca^{2+} in hydroxyapatite forming lead-apatite after periods of 5–45 days [52]. In fact, it is possible that the protein may have a higher affinity for a Pb^{2+} substituted crystal since we have shown this to be the case with Pb^{2+} in solution.

Our data shows that the binding of osteocalcin to hydroxyapatite is a Ca^{2+} regulated process. More protein can bind to hydroxyapatite at concentrations of Ca^{2+} which are greater than 1 mM. It is possible that during bone mineralization there are local increases in Ca^{2+} which may be a signal for a conformational change and increased binding of osteocalcin to the bone crystal. We show that 1 μM Pb^{2+} added

to physiological Ca^{2+} (1 mM) produces a significant ($\sim 40\%$) increase in the number of sites occupied on the crystal over that observed with 1 mM Ca^{2+} alone. Therefore, low Pb^{2+} levels would mimic the signal at physiological Ca^{2+} concentrations and prematurely increase the binding of osteocalcin to the bone crystal.

Since bone is the major reservoir for total body Pb^{2+} even low blood levels can cause a significant accumulation of Pb^{2+} in bone over time. Bone lead is indicative of past exposure with a half-life of 16 years [53] and has been found to increase with age [54]. In addition, several studies have provided evidence that bone extracellular fluid is compartmentalized [55]. Depending on the level of free Pb^{2+} near the bone surface and/or the presence of Pb^{2+} in bone mineral, more osteocalcin may be bound to the bone in Pb^{2+} exposed individuals.

The biological relevance of our findings to the potential effects of lead intoxication on bone metabolism is limited by the fact that the function of osteocalcin is not precisely known. A role for osteocalcin in bone resorption is provided by experiments showing osteocalcin is a chemoattractant for osteoclast precursor cells [56,57] and may function as a resident signal for the recruitment and differentiation of bone resorbing cells [29,58]. More recent studies with the osteocalcin knockout mouse suggest a role in the regulation of bone remodeling [35,36]. Further support for this comes from the recent demonstration of osteocalcin receptors on osteoblasts [59]. A number of studies have shown that lead can affect bone remodeling in lead exposed animals as well as organ culture [9–13]. Osteocalcin may act either in free solution or bound to mineral to effect the biological response. If osteocalcin acts as a specific cellular signal, alterations in secondary structure or variation in the equilibrium between bone and blood may result in downstream physiologic changes. Increased binding of the protein to hydroxyapatite by μmolar amounts of lead may alter bone remodeling. These alterations could contribute to the decreased bone formation rate [10], increased bone resorption [9,11] and to reduced bone density observed in lead intoxicated animals [12]. It is possible, that the alteration of normal osteocalcin function in lead-intoxicated individuals could exacerbate age-related or postmenopausal bone loss. The increased rate of bone

turnover in elderly males and females and in postmenopausal women may provoke an increase in the release of stored lead [60]. In fact, a recent CDC report indicated that blood lead levels ($\geq 0.5 \mu\text{M}$) are highest in children from 1–5 years old and in individuals > 50 years old [1]. If osteocalcin is an important modulator of bone turnover, the interaction of lead with osteocalcin may promote the further release of lead from the bone.

There are no structural and functional studies concerning the effect of Pb^{2+} on Ca^{2+} binding proteins. The results reported in this paper show that at the molecular level, as at the cellular level, low Pb^{2+} levels can perturb Ca^{2+} regulated processes. Our results on the effect of Pb^{2+} on the structure and properties of osteocalcin may be relevant to other physiologically important Ca^{2+} -binding proteins. Low lead levels may affect calmodulin, which is activated by a signal of increased free Ca^{2+} , and contribute to neurological or other disorders observed in Pb^{2+} toxicity.

Acknowledgements

Support for this project was provided by NIH Grant ES-02030 to T.D., funding from the Division of Environmental Sciences, Children's Hospital at Montefiore (CHAM) at the Albert Einstein College of Medicine to T.D. and NIH Grant AR-38460 to C.G. The authors would like to thank Drs R.K. Gupta, M.E. Girvin and S.C. Almo for helpful discussions. The authors are grateful to Dr Ruth Angeletti and the Laboratory for Macromolecular Analysis and Proteomics for expertise in the synthesis and characterization of the synthetic bovine osteocalcin. The authors are also grateful to Shang Xu for technical assistance with the atomic absorption measurements.

References

- [1] J.L. Pirkle, R.B. Kaufmann, D.J. Brody, T. Hickman, E.W. Gunter, D.C. Paschal, *Environ. Health Sci.* 106 (1998) 745–750.
- [2] P.S.I. Barry, *Br. J. Ind. Med.* 38 (1981) 61–71.
- [3] P.S.I. Barry, *Br. J. Ind. Med.* 32 (1975) 119–139.
- [4] J.F. Rosen, M.E. Markowitz, P.E. Bijur, S.T. Jenks, L. Wieropolski, J.A. Kalef-Ezra, D.N. Slatkin, *Proc. Natl. Acad. Sci. USA* 86 (1989) 685–689.
- [5] M.L. Bleeker, F.E. McNeil, K.N. Lindgren, V.L. Masten, D.P. Ford, *Toxicol. Lett.* 77 (1995) 241–248.
- [6] R. Kim, C. Landrigan, P. Mossmann, D. Sparrow, H. Hu, *Am. J. Epidemiol.* 146 (1997) 586–591.
- [7] J. Schwartz, C. Angle, H. Pitcher, *Pediatrics* 77 (1986) 11281–11288.
- [8] R. Shukla, R.L. Bornschein, K.N. Dietrich, C.R. Buncher, O. Berger, P.B. Hammond, P.A. Succop, *Pediatrics* 84 (1989) 604–612.
- [9] G.M. Hass, W. Landerholm, A. Hemmens, *Am. J. Pathol.* 50 (1967) 815–845.
- [10] C. Anderson, M.R.C. Path, K.D. Danylchuk, *Lab. Invest.* 37 (1977) 466–469.
- [11] A. Escribano, M. Revilla, E.R. Hernández, C. Seco, J. González-Riola, L.F. Villa, H. Rico, *Calcif. Tissue Int.* 60 (1997) 200–203.
- [12] H.E. Gruber, T.V. Sanchez, F. Khalil-Manesh, H.E. Gonick, *J. Bone Miner. Res.* 7:S (1992) 174.
- [13] T. Miyahara, H. Komiyama, A. Miyanishi, M. Takata, M. Nagai, H. Kozuka, T. Hayashi, M. Yamamoto, Y. Ito, H. Odake, F. Koizumi, *Toxicology* 97 (1995) 191–197.
- [14] F.A.X. Schanne, T.L. Dowd, R.K. Gupta, J.F. Rosen, *Proc. Natl. Acad. Sci. USA* 86 (1989) 5133–5135.
- [15] F.A.X. Schanne, T.L. Dowd, R.K. Gupta, J.F. Rosen, *Biochim. Biophys. Acta* 1054 (1990) 250–255.
- [16] T.L. Dowd, J.F. Rosen, R.K. Gupta, *J. Biol. Chem.* 265 (1990) 20833–20835.
- [17] G.J. Long, J.F. Rosen, *Toxicol. Appl. Pharmacol.* 114 (1990) 63–70.
- [18] R.F. Klein, K.M. Wren, *Endocrinology* 132 (1993) 2531–2537.
- [19] G.J. Long, J.F. Rosen, J.G. Pounds, *Toxicol. Appl. Pharmacol.* 106 (1990) 270–277.
- [20] P.V. Hauschka, J.B. Lian, P.M. Gallop, *Proc. Natl. Acad. Sci. USA* 72 (1975) 3925–3929.
- [21] P.A. Price, A.S. Otsuka, J.W. Poser, J. Kristaponis, N. Ramam, *Proc. Natl. Acad. Sci. USA* 73 (1976) 1447–1451.
- [22] A.L. Boskey, S.C. Marks, *Calcif. Tissue Int.* 37 (1985) 287–292.
- [23] M. Weinreb, D. Shinar, G.A. Rodan, *J. Bone Miner. Res.* 5 (1990) 831–842.
- [24] E.M. Aarden, A.M. Wassenaar, M.J. Alblas, P.J. Nijweide, *Histochem. Cell Biol.* 106 (1996) 495–501.
- [25] A. Linde, M. Bhowan, W.C. Cothran, A. Hoglund, W.T. Butler, *Biochim. Biophys. Acta* 704 (1982) 235–239.
- [26] P.V. Hauschka, J.B. Lian, D.E.C. Cole, C.M. Gundberg, *Physiol. Rev.* 69 (1989) 990–1047.
- [27] J.W. Poser, P.A. Price, *J. Biol. Chem.* 254 (1979) 431–436.
- [28] D.J. DeFranco, J. Glowacki, K.A. Cox, J.B. Lian, *Calcif. Tissue Int.* 49 (1991) 43–50.
- [29] J. Glowacki, C. Rey, M.J. Glimcher, K.A. Cox, J. Lian, *J. Cell. Biochem.* 45 (1991) 292–302.
- [30] C. Chenu, S. Colucci, M. Grano, P. Zigrino, R. Barattolo,

- G. Zambonin, N. Baldini, P. Vergnaud, P.D. Delmas, A.Z. Zallone, *J. Cell Biol.* 127 (1994) 1149–1158.
- [31] W.H. Liggett, J.B. Lian, J.S. Greenberger, J. Glowacki, *J. Cell. Biochem.* 55 (1994) 190–199.
- [32] G.K. Hunter, P.V. Hauschka, A.R. Poole, L.C. Rosenberg, H.A. Goldberg, *Biochem. J.* 317 (1996) 59–64.
- [33] R.W. Romberg, P.G. Werness, B.L. Riggs, K.G. Mann, *Biochemistry* 25 (1986) 1176–1180.
- [34] A.L. Boskey, F.H. Wians, P.V. Hauschka, *Calcif. Tissue Int.* 37 (1985) 57–62.
- [35] P. Ducy, C. Desbois, B. Boyce, G. Pinero, B. Story, C. Dunstat, E. Smith, J. Bonadio, S. Goldstein, C. Gundberg, A. Bradley, G. Karsenty, *Nature* 382 (1996) 448–452.
- [36] A. Boskey, S. Gadaleta, C. Gundberg, S.B. Doty, P. Ducy, G. Karsenty, *Bone* 23 (1998) 187–196.
- [37] T.L. Dowd, J.F. Rosen, C.M. Gundberg, R.K. Gupta, *Biochim. Biophys. Acta* 1226 (1994) 131–137.
- [38] P.V. Hauschka, S.A. Carr, *Biochemistry* 21 (1982) 2538–2543.
- [39] F.H. Wians, K.E. Krech, P.V. Hauschka, *Magnesium* 2 (1983) 83–92.
- [40] C.M. Gundberg, P.V. Hauschka, J.M. Lian, P.M. Gallop, *Methods Enzymol.* 107 (1984) 516–544.
- [41] B. Merrifield, *Methods Enzymol.* 289 (1997) 3–13.
- [42] I. Annis, B. Hargittai, G. Barany, *Methods Enzymol.* 289 (1997) 198–220.
- [43] J.T. Yang, C.S.C. Wu, H.M. Martinez, *Methods Enzymol.* 130 (1986) 208–269.
- [44] P.J. Parsons, W. Slavin, *Spectrochim. Acta* 48B (1993) 925–939.
- [45] S. Lundberg, J. Bjork, L. Löfvenberg, L. Backman, *Eur. J. Biochem.* 230 (1995) 658–665.
- [46] R.A. Atkinson, J.S. Evans, P.V. Hauschka, B.A. Levine, R. Meats, J.T. Triffitt, A.S. Viridi, R.J.P. Williams, *Eur. J. Biochem.* 232 (1995) 515–521.
- [47] P.D. Delmas, D.D. Stenner, R.W. Romberg, B.L. Riggs, K.G. Mann, *Biochemistry* 23 (1984) 4720–4725.
- [48] P.V. Hauschka, P.M. Gallop, in: *Calcium Binding Proteins and Calcium Function*, Elsevier, New York, 1977, pp. 338–347.
- [49] P.A. Price, A.S. Otsuka, J.W. Poser, in: *Calcium Binding Proteins and Calcium Function*, Elsevier, New York, 1977, pp. 333–337.
- [50] M.I. Kay, R.A. Young, A.S. Posner, *Nature* 204 (1964) 1050.
- [51] E.F. Bries, J.C. Voegel, J.C. Barry, W.G. Waddington, R.M. Frank, *J. Appl. Cryst.* 19 (1986) 168–173.
- [52] Y. Moriwaki, K. Ida, R. Yamaga, *J. Chem. Soc. Jap.* 5 (1975) 801–807.
- [53] S. Skerfving, U. Nilsson, A. Schutz, L. Gerhardsson, *Scand. J. Work Environ. Health* 19 (Suppl. 1) (1993) 59–64.
- [54] C. Gamblin, C.L. Gordon, D.C.F. Meir, D.R. Chettle, C.E. Webber, *Appl. Radiat. Isot.* 45 (1994) 1035–1038.
- [55] J. Green, *Miner. Electrolyte Metab.* 20 (1994) 7–15.
- [56] J.D. Mallone, S.L. Teitelbaum, G.L. Griffen, R.M. Senior, A.J. Kahn, *J. Cell Biol.* 92 (1982) 227–235.
- [57] G.R. Mundy, J.W. Poser, *Calcif. Tissue Int.* 35 (1983) 164–168.
- [58] J. Glowacki, J.B. Lian, *Cell Differ.* 21 (1987) 247–254.
- [59] P.V.N. Bodine, B.S. Komm, *Bone* 5 (1999) 535–543.
- [60] E.K. Silbergeld, J. Schwartz, K. Mahaffey, *Environ. Res.* 47 (1988) 79–94.