Metal Ion Binding to Dog Osteocalcin Studied by ^{1}H NMR Spectroscopy†

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ABSTRACT: One-dimensional ^{1}H NMR was employed to study the effects of Ca^{2+} and Lu^{3+} binding on the apo and calcium-saturated forms of dog bone Gla protein (BGP, osteocalcin). Titration of apo dog BGP with Ca^{2+} in 20 mM NaCl showed spectral perturbations consistent with the binding of 5 mol equiv of calcium in the NMR slow-exchange limit. The first 2 Ca^{2+} equiv induced significant conformational changes in the apoprotein, binding cooperatively with a $K_{d1} \sim 5.0 \times 10^{-4}$ M and a Hill coefficient H = 2.3 in 20 mM NaCl. The last 3 equiv bound with a slightly weaker affinity and did not induce significant structural changes. Neither the affinity nor the stoichiometry of calcium binding was significantly altered at 150 mM NaCl. The addition of only 1 Lu^{3+} equiv to apo dog osteocalcin was sufficient to induce the same spectral perturbations as 2 Ca^{2+} ions. The addition of 2 Lu^{3+} equiv to calcium-saturated osteocalcin had little effect on its ^{1}H NMR spectrum, and BGP aggregated at $[Lu^{3+}]_o/[BGP]_o$ ratios greater than 2 in either the presence or absence of calcium. The spectrum of calcium-saturated osteocalcin was invariant at ≤55 °C (≤50 °C in 150 mM NaCl), after which the proton resonances shifted to frequencies more characteristic of apo BGP. Saturation with calcium somewhat stabilized the apo dog osteocalcin protein against conformational changes induced at pH extremes; apo BGP was stable at $6.0 \leq pH \leq 10$, and calcium-saturated BGP was stable at $5.8 \leq pH \leq 10$. Both our NMR and gel filtration data indicate that calcium-saturated osteocalcin exists as a dimer at both high and low protein concentrations. A conformational change in dog osteocalcin was thus induced by the cooperative association of Ca^{2+} to two high- affinity sites on the protein and stabilized by the association of 3 additional Ca^{2+} equiv. The results of our temperature and calcium binding studies were consistent with an estimated $K_{d1} \sim 5.0 \times 10^{-4}$ M for the two high-affinity sites. Lutetium induced the same structural changes in osteocalcin as calcium, but the two high-affinity Ca^{2+} binding sites did not have equal affinities for Lu^{3+}. The BGP:Ca^{2+} complex was unstable at the low pH conditions induced by osteoclasts during bone resorption, yet the osteocalcin protein retained a BGP:Ca^{2+}-like conformation at low pH. However, unlike the calcium-saturated form of the protein, osteocalcin was monomeric at low pH.

Bone mass results from the equilibrium of two activities: the resorption of old bone by osteoclasts and the formation of new bone by osteoblasts. Bone is a two-phase tissue composed of inorganic hydroxylapatite (HAP)[1] and an organic matrix which is 90% collagen. Of the non-collagenous matrix proteins, bone Gla protein (BGP, osteocalcin) and matrix Gla protein are vitamin K-dependent, but only osteocalcin is bone-specific [reviews: Lian and Gundberg (1988) and Hauschka et al. (1989)].

Osteocalcin is the most abundant non-collagenous protein in the extracellular bone matrix (Price et al., 1987; Hauschka et al., 1975). It is located primarily in this matrix, adsorbed to hydroxylapatite, but it is also present in plasma where its concentration is thought to reflect new synthesis by bone cells (Price et al., 1981; Hauschka et al., 1983). Serum osteocalcin levels correlate well with a number of metabolic bone disorders, and the protein is a chemoattractant for a number of cell types found adjacent to endosteal bone surfaces *in vivo* (Mundy & Poser, 1983; Malone et al., 1986; Lucas et al., 1988). Osteocalcin most probably functions as a signaling system which couples bone formation and resorption (Lian, 1988) and as an inhibitor of mineralization (Romberg et al., 1986; Price, 1987), but the mechanisms which could support these potential functions have yet to be determined.

Osteocalcins are a family of small, acidic proteins (p$I \sim$ 4.0) containing 46–50 amino acids [Colombo et al., 1993; reviews: Hauschka (1986) and Hauschka et al. (1989)]. These proteins bind calcium, have a high affinity for HAP, and are potent inhibitors of calcium phosphate precipitation from solutions supersaturated with calcium and phosphate (Price et al., 1976; Poser & Price, 1979). Conserved elements in all osteocalcin sequences include 2–3 Gla residues (γ17, γ21, and γ24; human and wallaby BGPs lack γ17) and one disulfide bond (C23–C29), which are required for Ca^{2+} binding (Poser & Price, 1979; Hauschka & Carr, 1982; Colombo et al., 1993). An Asp- and Glu-rich domain containing residues E40 and/or D30, E31 and D34 is also conserved. The sequence of dog osteocalcin is shown here (Colombo et al., 1992):

YLDSG LGAPV PYPDP LγPKR γVCγL

NPNCD ELADH IGFQE AYQRF YGPC

The BGP:Ca^{2+} complex has increased affinity for HAP (Hauschka & Carr, 1982) and interacts with phospholipid vesicles (Gendreau et al., 1989). Since the adsorption affinity of osteocalcin for HAP and/or membrane surfaces may be an important factor in the mineral dynamics of bone, a detailed knowledge of its metal ion binding properties is needed in order to gain insight into the biological function(s) of the protein.

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[1] Abbreviations: BGP, bone Gla protein (osteocalcin); CD, circular dichroism; COSY, chemical shift correlated spectroscopy; γ or Gla, γ-carboxyglutamic acid; Hz, hertz; HAP, hydroxylapatite; NMR, nuclear magnetic resonance; ppm, parts per million; UV, ultraviolet; $[Ca^{2+}]_o$, total calcium concentration; $[BGP]_o$, total osteocalcin concentration.

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Despite the high degree of sequence homology between species (Colombo et al., 1993), both the stoichiometry and affinity of osteocalcin proteins for Ca^{2+} have been reported to vary significantly between species. Equilibrium dialysis, gel filtration, and a variety of spectroscopic studies (CD, fluorescence, laser Raman, NMR, UV) have shown apo BGP proteins to bind anywhere from 1 to 7 mol of Ca^{2+}/mol of protein, depending on the protein source and the ionic strength, with K_d values ranging from 0.024 to 7 mM (Iwami et al., 1987; Hauschka & Gallop, 1977; Hauschka & Carr, 1982; Price et al., 1977; Dendramis et al., 1983; Svard et al., 1986; Prigodich et al., 1985; Gundlach & Voegeli, 1983). Similarly, species-specific increases in α-helix content, anywhere from 7 to 30%, are induced in osteocalcin proteins by calcium, although all of the apoproteins displayed little secondary structure in solution (Gundlach & Voegeli, 1983; Delmas et al., 1984; Hauschka & Carr, 1982; Iwami et al., 1987).

The association of a variety of other metal ions with osteocalcin proteins has similarly shown a species-specific range of affinities and stoichiometries (Hauschka & Gallop, 1977; Hauschka & Carr, 1982; Gundlach & Voegeli, 1983; Iwami et al., 1987). Among these, the lanthanide ions appear to be good calcium analogs for osteocalcin studies. Both Tb^{3+} and La^{3+} were shown to competitively displace Ca^{2+} from its binding sites in chicken osteocalcin at pH 7.6 (Hauschka & Gallop, 1977), and Tb^{3+} was observed to induce approximately the same degree of α-helical character in the protein as Ca^{2+}, suggesting that it binds in a similar manner (Hauschka & Carr, 1982). Titration of the apoprotein with the fluorescent lanthanide Tb^{3+} revealed an "induction region" where no emission occurred during the addition of the first 2 equiv of Tb^{3+}. An abrupt 200-fold increase in fluorescence accompanied the addition of 2–3 more equiv. Beyond 5 mol of Tb^{3+}/mol of chicken osteocalcin, the fluorescent intensity was unchanged (Hauschka & Gallop, 1977). These results suggested that the first 2 Tb^{3+} equiv bound to osteocalcin much more tightly, associating with sites remote from Tyr residues. The induction of fluorescence emission upon addition of the last 3 Tb^{3+} equiv is consistent with the binding of lanthanide ions to sites at and/or near surface-exposed Tyr residues, sites which are distinct from those that bound the first 2 lanthanide equiv.

The differential binding of calcium and lanthanide ions to distinct types of calcium binding sites has also been observed with other calcium binding proteins, including the EF-hand calcium binding proteins calbindin and parvalbumin (Shelling et al., 1985a; Williams et al., 1984) and the sarcoplasmic reticulum ATPase (Squier et al., 1992). Calbindin and parvalbumin, for example, each bind 2 mol equiv of calcium with the same affinity, yet lutetium and ytterbium sequentially bind to these proteins in essentially the same manner (induce the same conformational changes) as calcium. The unique metal ion binding and spectroscopic properties of lanthanide ions have proven useful in determining the calcium binding properties specific to each distinct type of calcium binding site in these proteins, leading to a better understanding of how they function *in vivo* (Williams et al., 1986; Squier et al., 1992).

This article describes the use of 1D proton NMR to study the conformational changes in dog osteocalcin induced by both calcium and lutetium, a diamagnetic calcium analog. The results clearly show that dog osteocalcin binds 5 mol equiv of calcium. The association of the first 2 mol equiv of calcium was cooperative, and we demonstrate for the first time that all of the calcium-dependent conformational changes in osteocalcin are induced when these first 2 equiv bind to the protein. The subsequent binding of 3 additional calcium equiv to dog osteocalcin somewhat stabilized the structure already formed, but did not induce any further conformational changes in the protein. Lutetium induced the same conformational changes in the protein as calcium, and the two high-affinity sites of osteocalcin were shown to have quite different affinities for lanthanide ions. We are the first to directly observe this difference in lanthanide affinity and to determine that saturation of only one of these high-affinity calcium sites with lutetium is required to induce a calcium-dependent conformational change in the protein. The effects of temperature, pH, and ionic strength on the conformations of apo and calcium-saturated dog BGP and on the stability of the BGP:Ca^{2+} complex are described. We are also the first to report that the calcium-saturated osteocalcin protein exists as a dimer in solution.

These studies form the background for a comparative analysis of apo, partially/fully saturated, and low pH states of the protein, the results of which will provide detailed information as to how changes in structure upon ion binding are related to the biological properties of osteocalcin, including the increased affinity of the BGP:Ca^{2+} complex for HAP and cell surfaces. They also provide the essential information needed to initiate studies on the corresponding 3D solution structures of osteocalcin, including the calcium stoichiometries and affinities of the dog protein in both the neutral pH environments of serum and the low pH environment of bone induced by osteoclasts during bone resorption.

MATERIALS AND METHODS

Protein Samples. Dog osteocalcin was purified as described previously (Colombo et al., 1993). Approximately 4 mg of acid-extracted apo BGP protein was first neutralized by adding 30 μL of 1 M NH_4HCO_3, lyophilized, and then preexchanged twice in 300 μL of 99.9% D_2O before dissolution in 700 μL of one of two buffers: (1) 30 mM imidazole-d_4, 20 mM NaCl, pH 6.8 in D_2O; (2) 20 mM Tris-d_{11}, 150 mM NaCl, pH 7.4 in D_2O. In order to eliminate Ca^{2+} contamination, the D_2O used to exchange the apoprotein, and to make up the deuterated NMR sample buffers and stock metal ion solutions used in these studies, was pretreated with Chelex-100 (Shelling, 1984). Similarly, all glassware was treated (15 min) with 2% dimethyldichlorosilane (Sigma Chemical Company) in chloroform, followed by extensive washing with distilled water, prior to use. The final protein concentrations ($\sim$0.5 mM) were determined by amino acid analysis.

pH Measurements. The pH measurements were made with an Ingold microelectrode (Model 0300-04) attached to a Radiometer 24 pH meter; the pD values quoted are those observed and were not corrected for the deuterium isotope effect on the glass electrode. Values above pD 11 were not corrected for Na^+ interference and thus cannot be considered to be more accurate than $\pm$0.2 pH unit (Snyder et al., 1975). Electrode standardization was achieved prior to each measurement with standard, noncolored buffers (Fisher). pH adjustments were made by the addition of small volumes of 0.5 M DCl and/or 0.5 M NaOD (MSD isotopes). The sample pH was measured immediately prior to data acquisition. The total volume change during the pH titrations was $\leq$9%.

Stock Metal Solutions. Stock Lu^{3+} and Ca^{2+} solutions were prepared by weight from the corresponding chlorides, which had been dried overnight at 80 °C under bench vacuum. The final concentrations of these solutions in 99.9% D_2O were determined by titration with EDTA (Shelling et al., 1983,

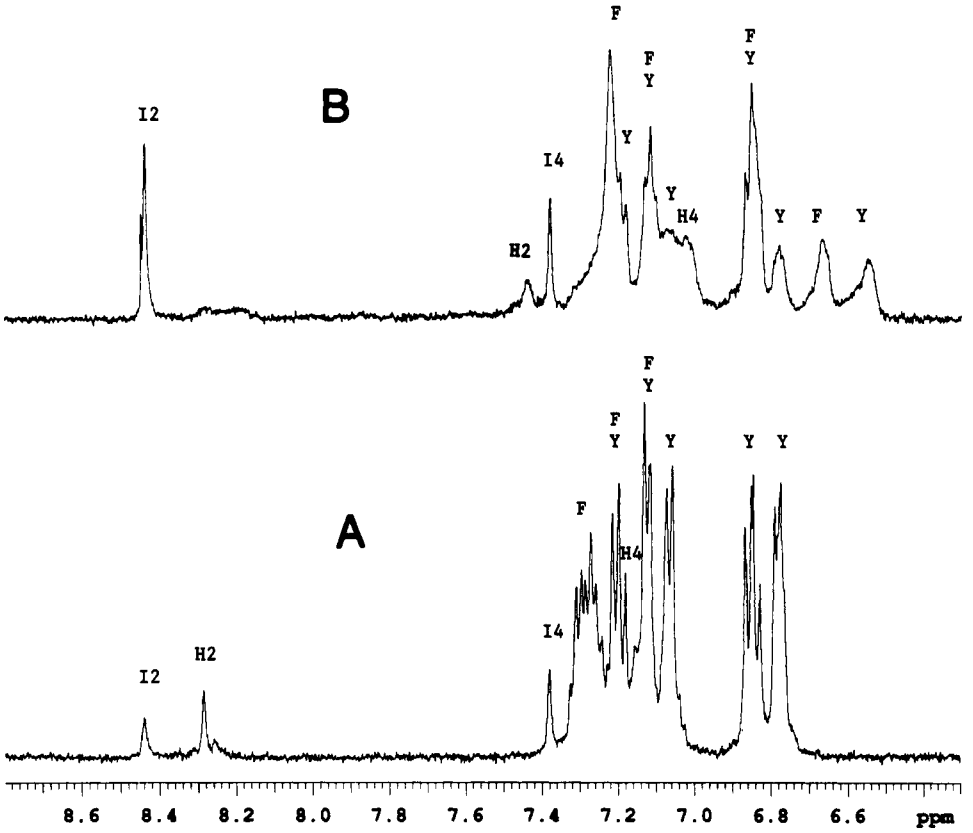

FIGURE 1: ^{1}H NMR spectra (aromatic region) of dog osteocalcin in the absence and presence of calcium. (A) Spectrum of 0.5 mM apo dog osteocalcin in 30 mM imidazole-d_4, 20 mM NaCl, pH 6.8 at 25 °C. (B) Calcium-saturated BGP ($[Ca^{2+}]_o/[BGP]_o = 10$) spectrum in the same buffer. The Tyr, Phe, and His assignments shown were determined by 2D NMR (COSY, data not shown); the imidazole (I) buffer resonances were assigned by comparison with 1D ^{1}H NMR spectra of buffer acquired in the absence of osteocalcin.

1984; Shelling, 1984). The total volume change during the Ca^{2+} and Lu^{3+} titrations was ≤8%.

NMR Spectra. The NMR spectra were obtained on a Varian VXR-500S NMR spectrometer operating in the Fourier transform mode and equipped with quadrature detection. The samples were run at 298 K (25 °C), and all samples were temperature-equilibrated for 15 min prior to data acquisition. The acquisition parameters were 8K data points (zero-filled to 16K for plotting data), a sweep width of 5000 Hz, and a 90° pulse width. Chemical shift values are relative to the major resonance of an internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) reference (0 ppm). Unless otherwise specified, the spectra represent 128 acquisitions that were resolution-enhanced with a Lorentzian to Gaussian conversion of 0.3 Hz. The effects of temperature on the apo and calcium-saturated proteins were measured over a range of 10–80 °C.

Gel Filtration. Before loading, all standards and protein samples were suspended in 500 μL of running buffer consisting of 30 mM imidazole, 20 mM NaCl, ± 5 mM $CaCl_2$, pH 6.8 (the same buffer used in the NMR experiments). Gel filtration experiments were performed on Econo-Columns (Bio-Rad Laboratories, Inc.; 1.0-cm i.d., 50-cm length, 40-mL bed volume, 30-mL/h flow rate, 1-mL fraction volume) using Bio-Gel P-10 resin (Bio-Rad, 100–200 mesh; 1500–20000 fractionation range). Four experiments were run, one each for calcium-saturated (Ca^{2+}/BGP = 10, the same ratio used in the NMR studies) and apo BGP at both high (0.5 mM) and low (71 μM) protein concentrations. The columns were pretreated with dimethyldichlorosilane, as described above. The resin was allowed to swell overnight in buffer, decanted five times from fresh buffer, and degassed prior to use; running buffers were also degassed. Molecular weight standards included the globular proteins aprotinin (MW 6500) and cytochrome C (MW 12 400), purchased from Sigma, as well as calbindin (MW 8500) and calmodulin (MW 16 700), which were generous gifts from Walter Chazin (Scripps Research Institute) and Tom Vanaman (University of Kentucky), respectively. The void volumes were determined with blue dextran. In order to ensure that residual calcium contamination did not affect the results of the apoprotein experiments, separate columns were used for experiments performed in the presence and absence of calcium.

RESULTS

Calcium-Induced Conformational Changes in Osteocalcin. The aromatic resonances of apo and calcium-saturated dog BGP are shown in Figure 1; these arise from the four Tyr, two Phe, and one His amino acid residues in the protein (see sequence above). Neither the 3,5 (6.70–6.90 ppm) nor the 2,6 (7.00–7.24 ppm) resonances of the four Tyr residues in apo BGP were equivalent, indicating that the apoprotein had some tertiary structure (Figure 1A). These aromatic resonances were significantly perturbed when the protein was saturated with calcium (Figure 1B). Upfield-shifted (shifted to lower frequency) Tyr and Phe resonances appear at 6.5–6.7 ppm, accompanied by a concomitant decrease in the Tyr 3,5 and 2,6 resonance intensities at 6.77 and 7.06 ppm, respectively, and in the Phe resonances at 7.12 and 7.22 ppm. Similarly, the broad Phe envelope of resonances at 7.23–7.35 ppm in apo BGP was observed to coalesce into a single broad resonance centered at 7.22 ppm. The H2 and H4 resonances of His were also significantly perturbed, with H2 shifting from 8.34 to 7.44 ppm and H4 from 7.18 to 7.02 ppm upon saturation with calcium. Distinct resonances characteristic of free and bound osteocalcin were thus observed in the apo

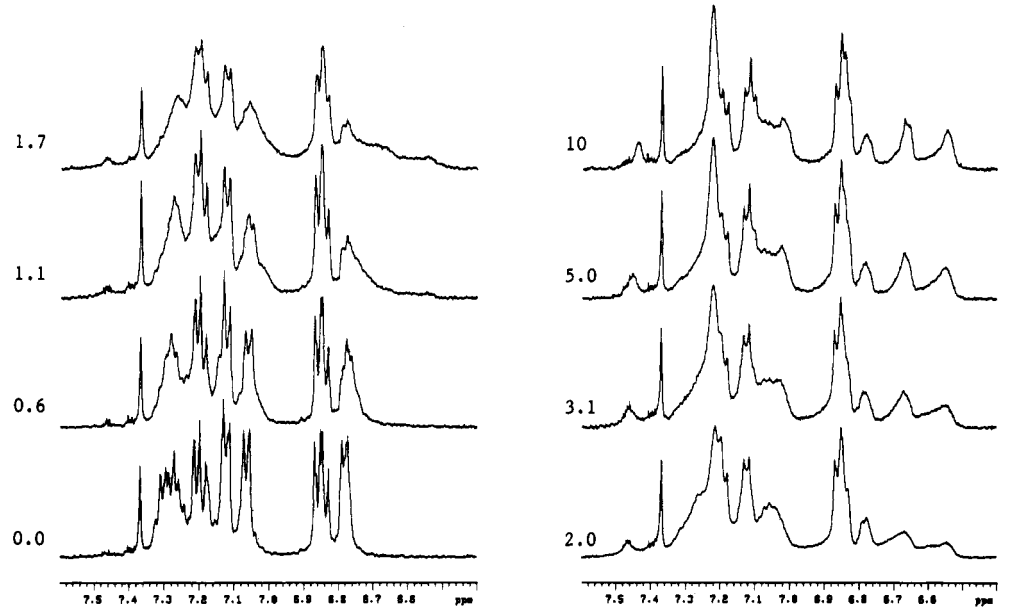

FIGURE 2: ^{1}H NMR spectra (aromatic region) acquired during a titration of apo BGP with Ca^{2+}. The sample was 0.5 mM dog osteocalcin in 30 mM imidazole-d_4, 20 mM NaCl, pH 6.8 at 25 °C. The $[Ca^{2+}]_o/[BGP]_o$ ratios are shown to the left of each spectrum. The signal at 7.37 ppm is from the imidazole H4 proton of the buffer (I4 in Figure 1).

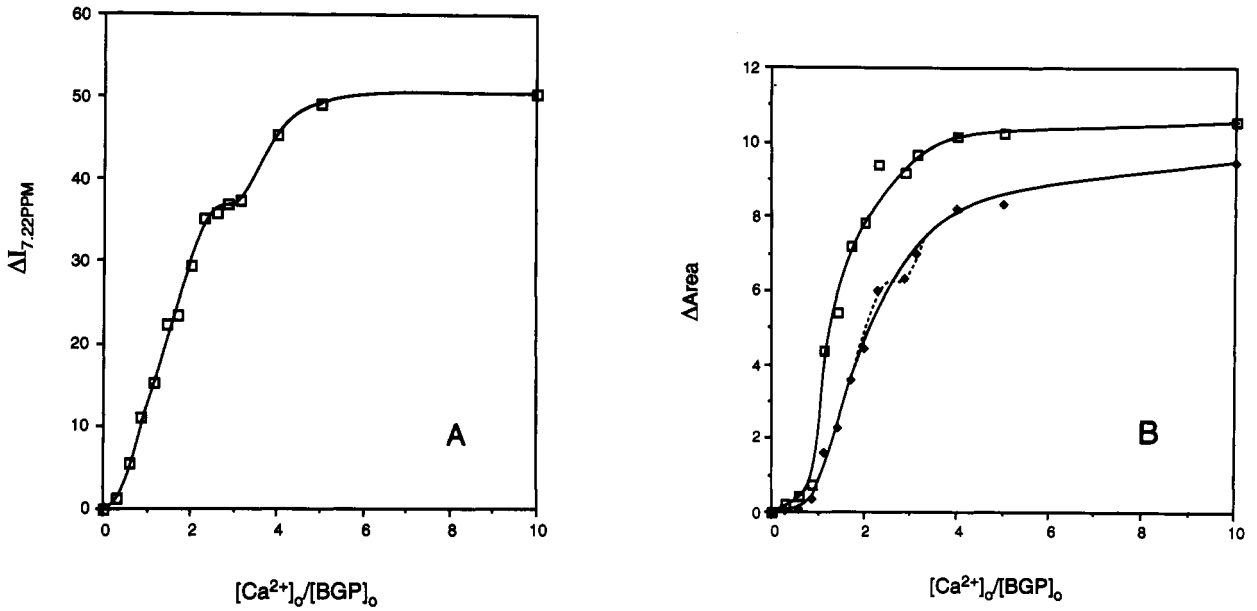

FIGURE 3: Plots of the change in intensity or area (in arbitrary units) of Phe and Tyr resonances in osteocalcin as a function of the $[Ca^{2+}]_o/[BGP]_o$ ratio. The data were taken in part from the spectra shown in Figure 2. (A, top) Intensity of the Phe 7.22 ppm resonance. (B, bottom) Areas of the upfield-shifted resonances of F (6.68 ppm, □) and Y (6.56 ppm, ◆) An alternate curve for the latter, which includes an inflection point similar to that found in part A, is indicated by a dotted line.

and calcium-saturated BGP spectra, indicating that the exchange between the liganded and unliganded forms of the dog protein was slow enough on the NMR time scale for the NMR slow chemical exchange condition to pertain (Kaplan & Fraenkel, 1980). These results showed that dog osteocalcin undergoes a major conformational change when it associates with calcium and that its calcium affinity is relatively strong ($K_d \leq 10^{-4}$ M).

The NMR spectra acquired during a titration of apo dog BGP with calcium are shown in Figure 2. Up to a $[Ca^{2+}]_o/[BGP]_o$ ratio of 2, the aromatic resonances of apo dog BGP shift significantly and broaden slightly. The most upfield Y3,5 and Y2,6 resonance pairs in the apoprotein spectrum, at 6.78 and 7.06 ppm, respectively, as well as the Phe envelope at 7.23–7.35 ppm, broaden and decrease in intensity up to a $[Ca^{2+}]_o/[BGP]_o$ ratio of 1. From $[Ca^{2+}]_o/[BGP]_o$ ratios of 1–2, new resonances characteristic of the calcium-saturated state of dog osteocalcin appeared, including upfield-shifted Tyr and Phe resonances and the narrowed Phe resonance centered at 7.22 ppm (Figures 1B and 2). The H2 and H4 resonances of His shifted to their calcium-saturated frequencies (Figure 1B), and upfield-shifted methyl resonances also became apparent (data not shown). These results show that apo dog osteocalcin undergoes a significant conformational change when it associates with these first 2 calcium equiv.

At $[Ca^{2+}]_o/[BGP]_o$ ratios of 2–5, there was little additional change in the spectrum, aside from a small increase in the intensity and area of these resonances. Thus these 3 additional calcium equiv induce no further conformational changes in the protein. Although the resonances perturbed by Ca^{2+} were somewhat broader than those of the apoprotein, all of the spectral changes observed when calcium was added were in

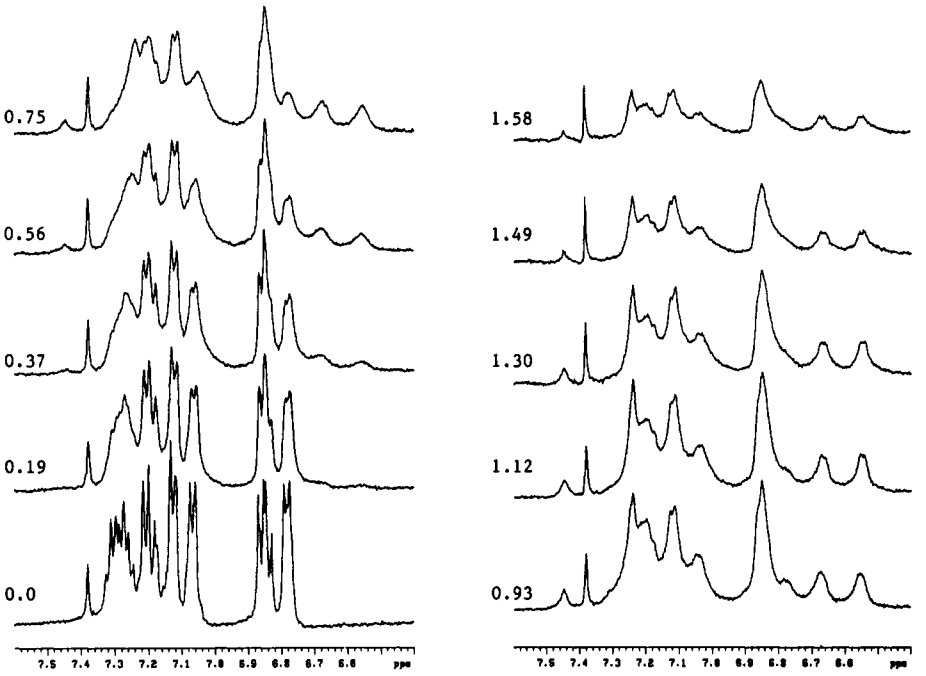

FIGURE 4: ^{1}H NMR spectra (aromatic region) acquired during a titration of apo BGP with Lu^{3+}. The sample was 0.5 mM dog osteocalcin in 30 mM imidazole-d_4, 20 mM NaCl, pH 6.8 at 25 °C. The $[Lu^{3+}]_o/[BGP]_o$ ratios are shown to the left of each spectrum. The signal at 7.37 ppm is from the imidazole buffer H2 proton (I2 in Figure 1).

the NMR slow-exchange limit, indicating that calcium bound fairly tightly to the protein. The slight narrowing of line widths observed when the last 3 calcium equiv associate with the protein indicates that its affinity for the first two calcium aliquots has increased somewhat; thus these last 3 equiv appear to stabilize the structure induced by the first 2 equiv. At $[Ca^{2+}]_o/[BGP]_o$ ratios ≥ 5, no additional spectral changes were observed, indicating that no more than 5 calcium equiv associate with apo dog BGP. Since all three of the four previous studies on osteocalcin proteins from other species showed calcium binding stoichiometries of 5–6, it is unlikely that additional calcium equivalents (≥ 5) associated with dog BGP in a manner that was spectroscopically silent (i.e., bound to the Ca^{2+}:BGP complex already formed, but induced no further observable changes in the NMR spectra).

Calcium Binding to Dog Osteocalcin Is Cooperative. Plots of the calcium-induced perturbations in the dog osteocalcin spectra are shown in Figure 3A,B. The data for the Phe resonance intensity at 7.22 ppm as a function of $[Ca^{2+}]_o/[BGP]_o$ are shown in Figure 3A. The solid line represents the sum of two curves: one for the binding of the first 2 Ca^{2+} equiv to the apoprotein and the second for binding of three additional calcium ions. The first curve is clearly sigmoidal, indicating that the first 2 Ca^{2+} equiv bind with positive cooperativity. A $K_d = \sim 5 \times 10^{-4}$ M was determined from the midpoint of this curve, with a Hill coefficient of 2.3 (Hill plot, data not shown). Since the inflection point between the two curves is so small, the affinity of the last 3 calcium equiv for dog BGP is only slightly weaker than that of the first 2. The presence of this inflection point, coupled with a lack of sufficient data points in the second curve, makes it difficult to determine from this data whether the last 3 calcium equiv also bound cooperatively. Corresponding plots of the areas of the two most upfield-shifted aromatic Tyr and Phe resonances as a function of added calcium are shown in Figure 3B. Although the sigmoidal nature of the first part of the binding curves is also readily apparent in this data, the transition midpoint seen in Figure 3A at $[Ca^{2+}]_o/[BGP]_o \approx 2$ is not as clearly defined. The assessment of the presence or absence of cooperativity using spectroscopic methods is thus highly dependent on the number of data points acquired and the molecular "probe" used (i.e., which amino acid and/or structural motif is being monitored), and NMR may have an advantage over other spectroscopic techniques because it can simultaneously monitor a large number of probes in osteocalcin.

Calcium Binding at Physiological Ionic Strength. At 150 mM NaCl, the spectrum of apo dog BGP was essentially the same as that at 20 mM NaCl, except that the H4 and H2 resonances of His shifted ~0.2 ppm upfield. The spectral perturbations observed upon the addition of increasing amounts of calcium to apo BGP in 150 mM NaCl (data available as supplementary material) were essentially the same as those observed at 20 mM NaCl (Figure 2), except that the most significant spectral changes ceased at a $[Ca^{2+}]_o/[BGP]_o$ ratio of ~2.3 rather than ~2. Thus, the affinity of the protein for the first 2 calcium equiv, and the calcium-dependent conformational change they induce in the protein, was not significantly affected by ionic strength.

Binding Properties of the Calcium Analog Lutetium. Titration of apo dog BGP with Lu^{3+} showed essentially the same spectral perturbations induced by calcium, except that they occurred over a metal ion/BGP ratio of 0–1 (Figures 4 and 5) rather than 0–2 as observed with calcium. Thus only 1 Lu^{3+} equiv was required to induce the same spectral perturbations induced by 2 equiv of calcium. Plots of this lanthanide binding data are shown in Figure 5. The data for the upfield-shifted Tyr resonance appear to be sigmoidal and thus indicative of cooperative binding. Further additions of Lu^{3+} visibly precipitated the protein ($[Lu^{3+}]_o/[BGP]_o \geq 1.12$), resulting in additional line-broadening and a net decrease in the areas of all the resonances in the NMR spectra (Figures 4 and 5). However, no significant changes in the chemical shifts and relative intensities of the resonances were observed during aggregation, indicating that the soluble Lu^{3+}:BGP complex we were still observing was stable. In conjunction with the results of our calcium binding studies, this lanthanide-induced aggregation would appear to result from the binding of free lanthanide ions to the protein surface, as observed with

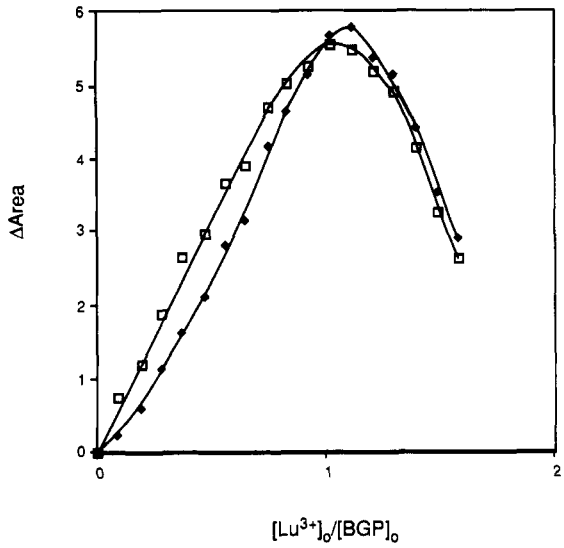

FIGURE 5: Plot of the change in area (arbitrary units) of the upfield-shifted F (6.68 ppm, □), and Y (6.56 ppm, ◆) resonances of apo dog osteocalcin as a function of the $[Lu^{3+}]_o/[BGP]_o$ ratio. The data were taken in part from the spectra shown in Figure 4.

calbindin (Shelling et al., 1985a). The extent of lanthanide-induced aggregation increased as the $[Lu^{3+}]_o/[BGP]_o$ ratio was raised above 1 and was essentially complete at ratios >1.6. In contrast, the addition of 2 Lu^{3+} equiv to Ca^{2+}-saturated osteocalcin ($[Ca^{2+}]_o/[BGP]_o = 10$) had no effect on the spectrum, although the further addition of Lu^{3+} to this sample ($[Lu^{3+}]_o/[BGP]_o > 2$) also induced protein aggregation (data not shown).

If we assume that the calcium binding site affinities of dog osteocalcin are greater for Lu^{3+} than for Ca^{2+}, as expected on the basis of its larger charge/radius ratio and similar size (Shannon, 1976), and from the results of earlier lanthanide binding studies of chicken osteocalcin (Hauschka & Gallop, 1977), these results indicate that the second Lu^{3+} equivalent induced no further structural changes in the protein, that it bound cooperatively but much more weakly than the first Lu^{3+} equivalent, and that association of Lu^{3+} to the second high-affinity calcium binding site was stabilized by the presence of calcium at the low-affinity sites. A lower Lu^{3+} affinity of the second high-affinity calcium binding site would thus result in free Lu^{3+} ions being added to the apoprotein, which could then bind to the protein surface and induce aggregation. However, calcium saturation of the three low-affinity calcium binding sites prior to the addition of lutetium could stabilize the $[Lu^{3+}]_o/[BGP]_o = 2$ complex. This interpretation is consistent with our calcium titration results, since addition of the last 3 calcium equiv to the low-affinity sites somewhat increased the affinity of calcium binding to the two high-affinity sites. However, from these studies we cannot determine whether aggregation in the presence or absence of calcium at $[Lu^{3+}]_o/[BGP]_o > 2$ was induced by excess Lu^{3+} binding nonspecifically to the protein surface and/or to the low-affinity calcium sites.

Temperature Stability of the Ca^{2+}:BGP Complex. The chemical shifts and line widths of Ca^{2+}-saturated dog BGP at 20 mM NaCl narrowed and shifted only very slightly from 10 to ~55 °C, after which they broadened somewhat and began to shift toward apoprotein frequencies as calcium dissociated from the protein (Figure 6). In 150 mM NaCl, the proton resonances began to shift toward their apoprotein equivalents at ~50 °C (data available as supplementary material). These temperature effects were fully reversible from 10 to 80 °C at both 20 and 150 mM NaCl. Since no significant resonance line-broadening was observed prior to BGP:Ca^{2+} complex dissociation, the exchange rate between calcium-free and calcium-bound BGP protein under these conditions was slow on the NMR time scale. This observation supports our earlier conclusion that dog osteocalcin has a relatively high affinity for calcium. Also, the BGP:Ca^{2+} complex was only very slightly less stable at high ionic strength, consistent with the very small decrease in calcium affinity we observed for dog osteocalcin at 150 mM NaCl.

pH Stability of the Apo Osteocalcin and the Ca^{2+}:BGP Complex. The pH dependence of apo dog BGP at 20 mM NaCl is illustrated in Figures 7 and 8. The spectrum of the apoprotein aromatic resonances changed significantly at pH ≤6.5, where upfield-shifted resonances similar in character to those induced by calcium at neutral pH (Figures 1 and 2) were observed, and the downfield Phe resonance envelope at 7.23–7.35 ppm narrowed similarly. Dog osteocalcin thus takes on a calcium-like conformation at low pH. No data were acquired below pH 5, where the osteocalcin solutions became very turbid. At pH 7–9, the aromatic resonances of the apoprotein were not significantly altered, aside from the shift of the H4 resonance of His as it deprotonated between pH 6–9 (Figure 8; the H4 resonance of His is clearly visible at 6.97–6.92 ppm, pH 8–9). From pH ~9 to ~10, the Tyr resonances shifted upfield slightly, and then very significant shifts were observed between pH 10 and 11.5. The midpoint of this latter transition yields an estimated pK_a of 10.6, which is unusually high for Tyr. At pH ≥11.5, there was no further change in the spectrum and the Tyr resonances were still inequivalent. Thus the protein retained some tertiary structure, even at high pH.

In the presence of calcium, the upfield-shifted Tyr and Phe resonances characteristic of calcium-saturated dog osteocalcin were stable from pH 6.5 to 5.8, but started to shift downfield (to higher frequency) at pH ≤5.6, taking on spectral characteristics more similar to those of the apoprotein at low pH (data available as supplementary material). At elevated pH levels, the upfield-shifted Tyr and Phe resonances titrated in the same pH range as that described for the apoprotein. However, the upfield-shifted resonances which characterize the calcium-bound state of the protein were clearly perturbed at pH 10–11.6 and were essentially absent at pH ≥12, indicating that the BGP:Ca^{2+} complex dissociated at pH >10 (data available as supplementary material).

Osteocalcin Is a Dimer in the Presence of Excess Calcium. Our temperature studies indicated that the resonance line-broadening observed upon the addition of calcium did not arise from chemical exchange processes (Figure 6). Since the signal/noise ratios of the spectra were essentially unaltered by the addition of calcium (Figure 1), we also ruled out nonspecific aggregation as the source. However, since the formation of soluble oligomeric proteins was still a possibility, we ran gel filtration studies on the protein. Under the conditions used in the calcium-saturated osteocalcin NMR experiments (0.5 mM BGP, 20 mM NaCl, 30 mM imidazole, and 5 mM $CaCl_2$), one major peak eluted from a P-10 column (Figure 9). This peak had an apparent molecular weight of 12 200, with a very small shoulder at 7400. In the same buffer without calcium, the 0.5 mM protein sample eluted with an apparent molecular weight of 13 100. At a lower protein concentration, one more typical of those used for CD, fluorescence, and other spectroscopic experiments (71 μM, 0.4 mg/mL), the calcium-saturated protein eluted with an

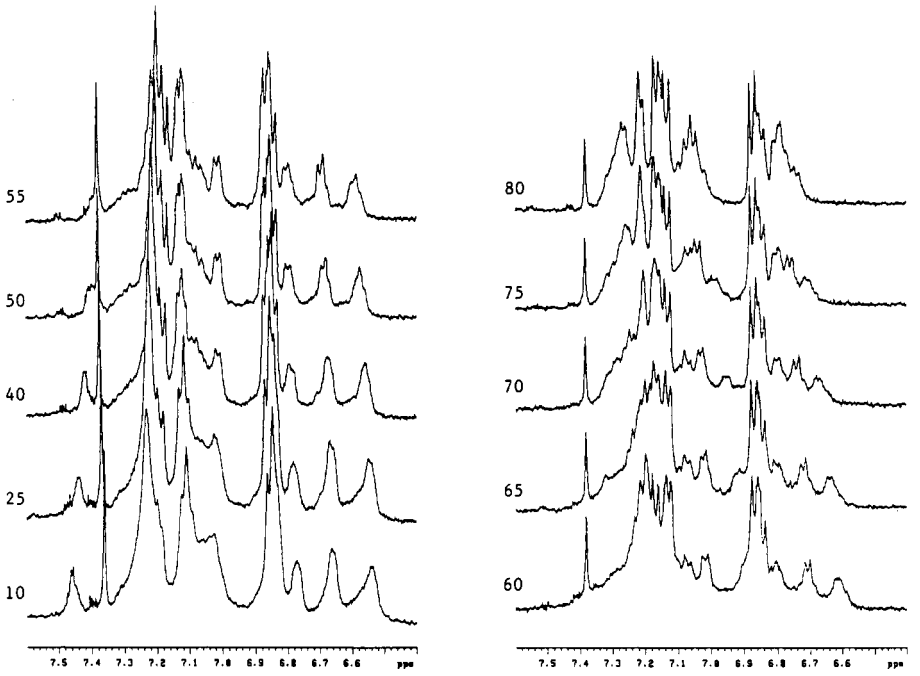

FIGURE 6: Effects of temperature on the proton NMR spectrum of 0.5 mM dog osteocalcin in 5 mM $CaCl_2$, 30 mM imidazole-d_4 and 20 mM NaCl, pH 6.8 ($[Ca^{2+}]_o/[BGP]_o = 10$). The temperature (°C) is shown to the left of each spectrum.

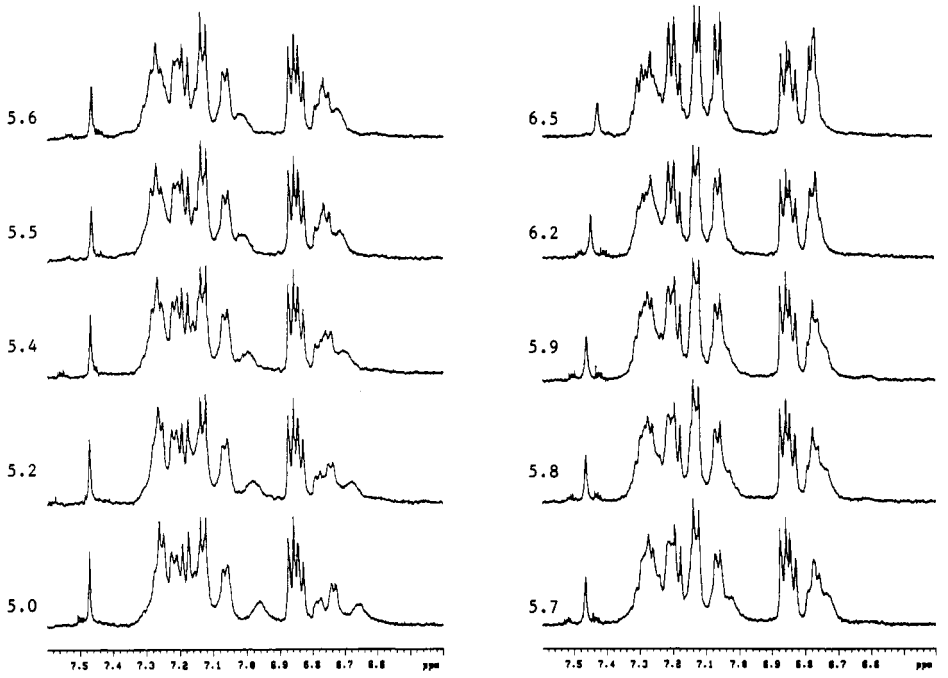

FIGURE 7: Proton NMR spectra of apo dog osteocalcin at pH ≤6.5. The sample consisted of 0.5 mM dog BGP in 30 mM imidazole-d_4 and 20 mM NaCl. The pH values are shown to the left of each spectrum.

apparent molecular weight of 11 800 (very small shoulder at 6000), the apoprotein with an apparent MW of 13 000 (data not shown).

Earlier gel filtration studies also showed that apo chicken BGP protein migrated with a high apparent molecular weight (Hauschka & Gallop, 1977), yet molecular weight determinations performed by sedimentation equilibrium and SDS gel electrophoresis experiments on both bovine and chicken osteocalcin clearly showed that these apoproteins were monomers (Price et al., 1976; Hauschka & Gallop, 1977). Thus, apo osteocalcin must exist in an extended conformation in solution, which leads to an anomalously high apparent molecular weight determination by gel filtration.

However, the apparent molecular weight observed for calcium-saturated osteocalcin by gel filtration was lower than that of the apoprotein, indicating that BGP becomes more compact when it binds calcium. This result is in agreement with earlier electrophoresis studies, which showed that the mobility of chicken osteocalcin decreased 2-fold in the presence of calcium (Hauschka & Gallop, 1977), and with the significant upfield-shifted NMR resonances we have described herein for dog osteocalcin which suggest that the protein folds up sufficiently to form hydrophobic clusters when calcium is added. It is also consistent with a compact, globular model structure of chicken osteocalcin based on CD, immunochemical, and secondary structure prediction studies (Hauschka &

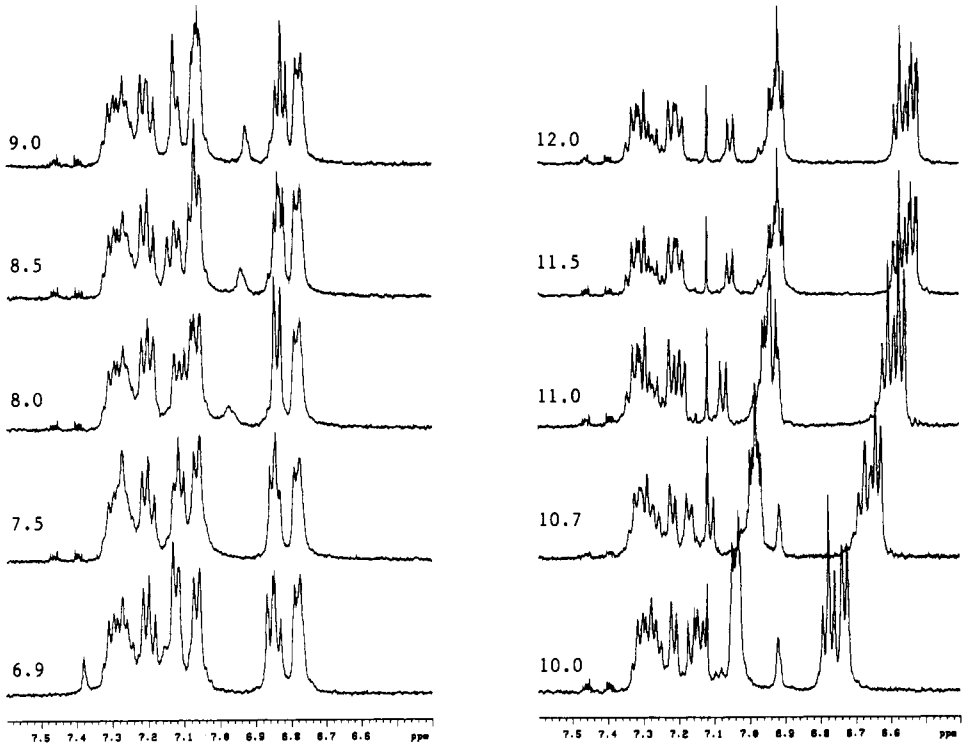

FIGURE 8: Proton NMR spectra of apo dog osteocalcin at pH ≥6.9. The sample consisted of 0.5 mM dog BGP in 30 mM imidazole-d_4 and 20 mM NaCl. The pH values are shown to the left of each spectrum.

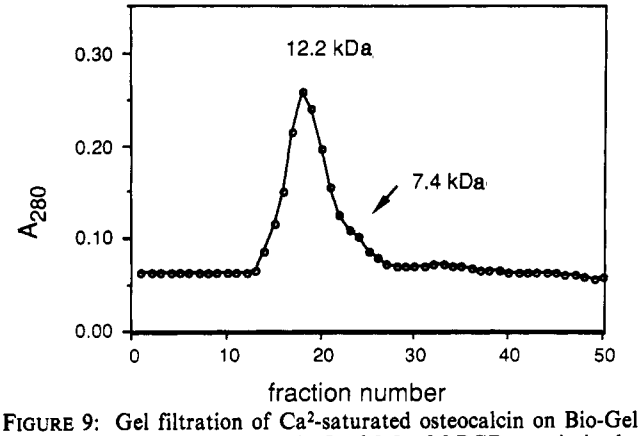

FIGURE 9: Gel filtration of Ca^2-saturated osteocalcin on Bio-Gel P-10 resin. The sample was 500 μL of 0.5 mM BGP protein in the column running buffer: 30 mM imidazole, 20 mM NaCl, and 5 mM $CaCl_2$, pH 6.8.

Carr, 1982), a model which apparently was also supported, at least in part, by some preliminary 2D NMR data on rabbit osteocalcin (Termine, 1988).

The line-broadening observed in our NMR spectra of calcium-saturated osteocalcin thus indicates that calcium binding induces self-association and, since the protein appears to adopt a more globular conformation in the presence of calcium, the apparent molecular weight determined by gel filtration shows that it associates to form a dimer. Although it is clear from our results that calcium-saturated dog osteocalcin was primarily a dimer under the conditions used for our gel filtration and NMR experiments, the very small shoulders observed at MW 6000–7400 were not observed similarly with the molecular weight standards. If these arose from the presence of a small amount of calcium-saturated monomer, then calcium binding in itself is sufficient to make the protein more compact.

Since both apo and calcium-saturated proteins were observed to migrate similarly at both the higher concentration used for NMR studies and a lower concentration more typical of that used for CD, fluorescence, and other spectroscopic techniques, we can compare our results directly with those of earlier studies which employed these other techniques.

DISCUSSION

Calcium Binding Properties of Osteocalcin Proteins. Our observation of significant alterations in the environments of Tyr and Phe residues upon Ca^{2+} binding to dog osteocalcin is consistent with CD, laser Raman, UV, and fluorescence spectroscopy data obtained for other osteocalcin proteins (Hauschka & Carr, 1982; Dendramis et al., 1983; Gundlach & Voegeli, 1983). Our ^1H NMR studies have shown that dog BGP binds 5 calcium equiv. The first 2 equiv bind cooperatively with a $K_{d1} = \sim 5 \times 10^{-4}$ M, and the next 3 equiv bind with slightly less affinity. These affinities and stoichiometries lie within the ranges reported for other osteocalcin proteins, which have been shown to bind 2 Ca^{2+} ions with relatively high affinity($K_d \approx 0.1$ mM) and/or ≥2–4 Ca^{2+} ions with lower affinity ($K_d \geq 3$ mM), depending on the species and ionic strength (Gundlach & Voegeli, 1983; Delmas et al, 1984; Svard et al., 1986, Hauschka & Gallop, 1977; Hauschka & Carr, 1982; Iwami et al., 1987). In contrast, Ca^{2+}-induced structural changes and/or Ca^{2+} affinity in other osteocalcin proteins have been shown to be relatively sensitive to ionic strength in the 0 –150 mM NaCl range (Price et al., 1977; Hauschka & Carr, 1982; Delmas et al., 1984). The new information deduced from these studies is that only the first 2 calcium equiv are required to induce the calcium-dependent conformational change in osteocalcin.

We are also the first to demonstrate experimentally, via the calcium-induced coalescence of Phe resonances into a single envelope at 7.22 ppm and the appearance of upfield-shifted Phe and Tyr resonances, that at least two Tyr and likely both Phe side chains of osteocalcin form a tight cluster upon Ca^{2+} complexation, as similarly observed by NMR for the EF-hand type calcium binding of protein calbindin (Shelling et

al., 1983; Skelton et al., 1990). It is interesting to note that, although an opportunity to form stabilizing hydrophobic clusters was suggested by the model structure proposed by Hauschka and co-workers for chicken osteocalcin (Hauschka & Gallop, 1982), this model only contained three metal ion binding sites.

Bovine osteocalcin is the only other BGP protein which has been reported to bind Ca^{2+} cooperatively (Gundlach & Voegeli, 1983), with a Hill coefficient (H = 1.5 at pH 7.2) similar to that we observed for dog osteocalcin (H = 2.3 at pH 6.8). The presence or absence of cooperativity could be a subtle species-specific effect, since dog and bovine BGP differ in overall sequence by only six amino acids (12%), while the chicken and frog (Iwami et al., 1987) osteocalcin proteins are less homologous overall. However, all of these osteocalcins display a high degree of homology in those sequence elements involved in calcium binding (Gla domain, disulfide bond, and possibly the Asp-Glu domain). Therefore, another possibility is that previous plots of binding data from other species were not sigmoidal because insufficient data points were collected to define completely the binding properties of the protein, particularly if the sigmoidal nature of the binding data was as subtle as that we observed for the 7.22 ppm Phe resonance of dog osteocalcin.

The only other ^1H NMR studies reported for osteocalcin were carried out on the bovine protein at pH 8.5 in the absence of salt (Prigodich et al., 1985). In contrast to our results with dog osteocalcin, the addition of calcium to bovine osteocalcin in these earlier studies resulted in a large degree of line-broadening. This observation suggested that the NMR intermediate chemical exchange condition pertained in these studies (Kaplan & Fraenkel, 1980), leading one to conclude that the bovine BGP:Ca^{2+} complex formed was very labile (weak binding, $K_d \geq 10^{-3}$ M). Also, no significant changes in the chemical shifts of the ^1H NMR resonances of bovine osteocalcin were observed when calcium was added, suggesting that calcium had little effect on the conformation of the bovine osteocalcin in solution. These results are inconsistent with spectroscopic and equilibrium dialysis studies previously carried out on osteocalcin proteins from bovine and other species, as discussed above, and also with our NMR studies of dog osteocalcin. It is unlikely that the discrepancies between our studies on dog osteocalcin and the NMR studies on the bovine protein were the result of a significant difference in the calcium affinities of these two proteins at pH 8.5, since our studies clearly showed that the dog BGP:Ca^{2+} complex was stable at this pH. There also appeared to be no significant difference in the tertiary structures of the corresponding apoproteins at pH 8.5, as evidenced by the similarity of their ^1H NMR spectra.

Calcium and Lanthanide Binding to Osteocalcin. Our calcium binding data on dog osteocalcin are consistent with terbium fluorescence studies of chicken osteocalcin, which showed that 5 lanthanide equiv bound to the apoprotein, two with high affinity and three with lower affinity (Hauschka & Gallop, 1977). We similarly observed the tight association of 2 Lu^{3+} equiv to dog osteocalcin, one of which induced a calcium-like conformation in dog osteocalcin; these results are consistent with CD studies of Tb^{3+} binding to the chicken BGP protein (Hauschka & Carr, 1982).

However, our Lu^{3+} binding studies of dog osteocalcin are the first to show that the two high-affinity calcium binding sites of osteocalcin are not identical. Saturation of only one of these sites with Lu^{3+} was sufficient to induce a calcium-dependent conformational change in dog osteocalcin, and since this conformationally sensitive site had a significantly higher affinity for Lu^{3+} than the second site, the two sites cannot be identical. Both Lu^{3+} and Ca^{2+} appear to bind osteocalcin in a cooperative manner, the only difference being that Lu^{3+} has a higher affinity than Ca^{2+} for at least one of the two high-affinity calcium binding sites. The differential binding of calcium and lanthanide ions within a single class (i.e., high affinity) of calcium binding sites has previously been observed for EF-hand type proteins, including calbindin and parvalbumin (Shelling et al., 1985a; Williams et al., 1984), and for the Gla domain of prothrombin, prothrombin fragment 1 (Marsh et al., 1981), but has not been reported for a bone Gla protein. Since the metal ion binding modes of osteocalcin, and Gla proteins in general, are so little understood, the similarity of the spectral changes induced by Ca^{2+} and Lu^{3+} and their differential binding properties to osteocalcin indicate that lanthanide ions will be useful calcium analogs for future studies on both partially and fully saturated states of BGP, as previously shown for the EF-hand calcium binding proteins calbindin and parvalbumin (Shelling et al., 1985a,b; Williams et al., 1986). The results of such studies should provide insights as to how osteocalcin, and Gla proteins in general, bind metal ions and how calcium binding and calcium-induced cooperativity mediate the biological activities of these proteins.

We were unable to observe the effects of additional Lu^{3+} equivalents ($[Lu^{3+}]_o/[BGP]_o > 2$) due to protein aggregation in both the presence and absence of excess calcium. Aggregation and/or anomalous spectroscopic data arising from nonspecific binding of excess lanthanide ions have been observed in NMR studies of EF-hand type calcium binding proteins (Shelling et al., 1985a). It is unclear in our studies whether aggregation was the result of lanthanide ions binding nonspecifically or specifically to the surface of the dog osteocalcin protein. The second possibility, in which the association of free lanthanide ions with the three low-affinity calcium binding sites of osteocalcin induces self-association, is an intriguing one because a model structure proposed for chicken osteocalcin suggests that its three Gla residues project in the same direction away from one face of a "Gla-helix" in such a way that they could interact directly with Ca^{2+} ions on the surface of hydroxylapatite (Hauschka & Carr, 1982). Since lanthanide-induced aggregation was not reported in the Tb^{3+} fluorescence studies of chicken osteocalcin, it may be a concentration dependent phenomenon.

Temperature Stability of Apo and Calcium-Saturated Osteocalcin. The chemical shifts and line widths of Ca^{2+}-saturated dog osteocalcin were essentially invariant up to ~55 °C, after which they broadened somewhat and began to shift toward apoprotein frequencies; the effect was reversible up to 80 °C (Figure 6). This result is consistent with an increased rate of exchange between the free and calcium-bound forms of dog osteocalcin, as a direct result of the protein's decreased affinity for calcium at elevated temperatures. The same basic trend was observed at 150 mM NaCl, except that the resonances shifted at the slightly lower temperature of ~50 °C (data available as supplementary material). These results verified that apo dog osteocalcin bound calcium in the NMR slow-exchange limit at physiological temperatures. They also suggest that the affinity of the protein for calcium is within the range $10^{-6} \leq K_d \leq 10^{-3}$ M, since the temperature stability observed was less than those of EF-hand type calcium binding proteins ($K_d \leq 10^{-6}$ M), and support our previous conclusion that the ionic strength of the medium has little effect on the inherent stability of the complex.

pH Stability of Apo and Calcium-Saturated Osteocalcin. The degree of tertiary structure in apo osteocalcin has been shown to result from the effects of electrostatic repulsion and the presence of the single disulfide bond (Hauschka & Carr, 1982). It is thus likely that the conformation of the apoprotein at neutral pH is dictated by the interactions between its closely spaced negative charges, which are known to disturb α-helices (Lewis & Bradbury, 1974), and that helical structures are free to form at lower pH when these negative charges are partially or totally neutralized. Apo dog osteocalcin undergoes a significant conformational change at pH $\leq$6.5 (Figure 7), and the spectral changes observed were similar to those induced by the addition of calcium. This result is consistent with earlier CD studies (Gundlach & Voegeli, 1983; Delmas et al., 1984) which showed that α-helix formation in apo bovine osteocalcin could be induced at low pH. However, the amount of helix induced in these studies was substantially less than that observed for calcium-saturated osteocalcin at neutral pH, yet it was clear from our studies on dog BGP that the overall conformation of the apoprotein at low pH was very similar to that of the calcium-saturated osteocalcin at neutral pH.

We were unable to carry out a complete low pH range study of apo dog osteocalcin because the protein was insoluble at $4 < pH < 5$, but the results we did obtain from pH 5 to 6.9 (Figure 7) were consistent with the transition midpoints of pH 5.7 (Hauschka & Gallop, 1977) and pH 6.1 (Gundlach & Voegeli, 1983) previously determined for chicken and bovine osteocalcin proteins, respectively. The insolubility of dog osteocalcin protein at $4 < pH < 5$ suggests that its isoelectric point lies within this range, which is significantly higher than that calculated for dog osteocalcin on the basis of its amino acid sequence. One or more amino acid residues in this protein thus have elevated pK_a values. The His resonance of apo dog osteocalcin titrated with an unusually high pK_a of $\sim$7.7 (Figure 8), as observed previously for bovine osteocalcin (pK_a = 7.8; Prigodich et al., 1985). The chemical shift of this resonance was also affected significantly by ionic strength. It is thus likely that this His residue directly interacts with one or more negatively charged side chains in the apoprotein.

The fact that we observed little change in the spectrum of apo dog BGP from pH 7.2 to 9.0 agrees with the results of CD studies on bovine BGP (Gundlach & Voegeli, 1983). The chemical shift changes observed for the Tyr resonances of both apo and calcium-saturated dog osteocalcin between pH 9 and 11 were consistent with the normal alkaline titration properties of these residues under both conditions. There was no evidence that calcium induced phenolate ion formation well below the normal pK_a of 10 for Tyr, as was previously suggested for chicken osteocalcin (Hauschka & Carr, 1982), or that one or more Tyr pK_a values were elevated by the presence of calcium, as apparently observed for the rabbit protein (Termine, 1988). Also, since the Tyr and Phe resonances were still disperse at pH 12, we can conclude from our studies that the dog apoprotein retained some tertiary structure even at high pH, consistent with the proposal that its tertiary structure is the result of charge repulsion.

The spectral similarities of the calcium-saturated protein at neutral pH and the apoprotein at low pH made analysis of the data acquired on the former at and between these points unambiguous. However, although it appeared that calcium somewhat hindered the protonation of one or more carboxyl groups in the protein, the dog BGP:Ca^{2+} complex was unstable at low pH. The instability of the dog BGP:Ca^{2+} complex at pH <6 is consistent with the pK_a = 5.1–6.1 values previously determined for chicken (Hauschka & Gallop, 1977) and bovine osteocalcin (Gundlach & Voegeli, 1983; Svard et al., 1986) and apparently also observed with rabbit osteocalcin (Termine, 1988). At pH $\geq$10, the characteristic upfield-shifted Tyr and Phe resonances of calcium-saturated BGP were perturbed, and they disappeared entirely at pH $\geq$11.6. Thus, we can also conclude from our studies that the BGP:Ca^{2+} complex was unstable at high pH.

These results clearly show that the dog BGP:Ca^{2+} complex was unstable at $6 \leq pH \geq 10$. Yet the conformation of the apoprotein at low pH was very similar to that of calcium-saturated osteocalcin at neutral pH. The effects of low pH on the conformation of dog osteocalcin and the stability of the BGP:Ca^{2+} complex may be physiologically significant, since osteoclasts induce local low pH environments during bone resorption (Arnett, 1990; Arnett & Dempster, 1990). However, the effects of lowered pH on the affinity of osteocalcin for hydroxylapatite, phospholipids, BGP-specific receptors, or other bone matrix proteins such as osteopontin (Ritter et al., 1992) have yet to be determined.

Dimerization of Osteocalcin in the Presence of Calcium. Our gel filtration and NMR studies indicate that calcium-saturated BGP is a dimer. Since the α-helical content of osteocalcin increases significantly in the presence of calcium, as well as at low pH, the protein could become partly amphipathic under certain conditions and favor a homophilic association. Similarly, the binding of cations such as Lu^{3+} and Ca^{2+} would reduce the net negative charge of the peptide, making the complex electrically neutral if not hydrophobic. However, at low pH we see an induction of conformation similar to that observed by the addition of calcium (Figure 7), but no significant changes in line widths and thus no evidence for dimerization. Analytical ultracentrifuge studies on chicken osteocalcin similarly have showed the apoprotein to be a monomer at low pH (Hauschka & Gallop, 1977). Therefore, it is unlikely that hydrophobic interactions alone are the cause of dimer formation; it may also be mediated by calcium.

Since only one set of upfield-shifted, calcium-bound ^1H NMR resonances was observed for each aromatic amino acid in these studies (Figures 1 and 2), the dimer should be symmetric. Under NMR slow-exchange conditions, the appearance of the broadened, upfield shifted aromatic and aliphatic resonances only at ratios of $1 < [Ca^{2+}]_o/[BGP]_o < 2$ (Figure 2) suggests that the monomer may dimerize upon addition of the second calcium equivalent. In contrast, only 1 equiv of lutetium was sufficient to induce dimer formation (Figure 4), which could result from its ability to form complexes with higher coordination numbers than calcium (Shannon, 1976). However, since the protein binds calcium cooperatively, and we cannot unambiguously differentiate between the inter- and intramolecular effects of metal ion binding from these studies alone, such models of BGP dimerization are highly speculative. Similarly, resonance assignments for the upfield-shifted aromatic protons cannot in themselves yield insights as to the overall fold of the protein, and a clearer understanding of the chain folding and pH effects during the BGP titrations will only be achieved when the 3D solution structures of the apo, partially/fully saturated, and low pH states of the protein have been determined. Analytical ultracentrifuge and additional spectroscopic studies of the structure and Ca^{2+} binding properties of the BGP:Ca^{2+} dimer are thus currently underway.

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SUPPLEMENTARY MATERIAL AVAILABLE

^{1}H NMR spectra of dog BGP, including Ca^{2+} titration spectra at 150 mM NaCl (Figure 1S), calcium-saturated spectra at 10–80 °C (Figure 2S), calcium-saturated spectra acquired at pH 5.0–6.5 (Figure 3S), and calcium-saturated spectra acquired at pH 7.0–12.0 (Figure 4S) (5 pages). Ordering information is given on any current masthead page.

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