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Calcium-Dependent α-Helical Structure in Osteocalcin[†]

Peter V. Hauschka[*] and Steven A. Carr

ABSTRACT: Osteocalcin is an abundant Ca^{2+}-binding protein of bone containing three residues of vitamin K dependent γ-carboxyglutamic acid (Gla) among its 49 (human, monkey, cow) or 50 (chicken) amino acids. Gla side chains participate directly in the binding of Ca^{2+} ions and the adsorption of osteocalcin to hydroxylapatite (HA) surfaces in vivo and in vitro. Osteocalcin exhibits a major conformational change when Ca^{2+} is bound. Metal-free chicken osteocalcin is a random coil with only 8% of its residues in the α helix as revealed by circular dichroism. In the presence of physiological levels of Ca^{2+}, 38% of the protein adopts the α-helical conformation with a transition midpoint at 0.75 mM Ca^{2+} in a rapid, reversible fashion which (1) requires an intact disulfide bridge, (2) is proportionally diminished when Gla residues are decarboxylated to Glu, (3) is insensitive to 1.5 M NaCl, and (4) can be mimicked by other cations. Tyr fluorescence, UV difference spectra, and Tyr reactivity to tetranitromethane corroborate the conformational change. Homologous monkey osteocalcin also exhibits Ca^{2+}-dependent structure. Integration of predictive calculations from osteocalcin sequence has yielded a structural model for the protein, the dominant features of which include two opposing α-helical domains of 9–12 residues each, connected by a β turn and stabilized by the Cys$_{23}$–Cys$_{29}$ disulfide bond. Cation binding permits realization of the full α-helical potential by partial neutralization of high anionic charge in the helical domains. Periodic Gla occurrence at positions 17, 21, and 24 has been strongly conserved throughout evolution and places all Gla side chains on the same face of one α helix spaced at intervals of $\sim$5.4 Å, closely paralleling the interatomic separation of Ca^{2+} in the HA lattice. Helical osteocalcin has greatly increased affinity for HA; thus, the Ca^{2+}-induced structural transition may perform an informational role related to bone metabolism.

Specific interaction between ionic calcium and proteins serves numerous important biochemical and biological functions in areas as diverse as muscle contraction, cell motility, fertilization, neurotransmission, blood coagulation, and hormonal regulation of metabolic pathways [see Kretsinger (1976) and references cited therein]. Ca^{2+} has been called the "second messenger" (Rasmussen & Goodman, 1977), and indeed the targets for its action are generally known to be proteins. This interaction may be classified according to the nature and affinity of the Ca^{2+} binding site and by the free Ca^{2+} concentration in the natural environment of the protein (Kretsinger, 1977). Certain enzymes require Ca^{2+} as a cofactor, and this cation is tightly bound in or near the active site [phospholipase A$_2$ (Verheij et al., 1980) and staphylococcal nuclease (Tucker et al., 1979)]. Others are merely stabilized by Ca^{2+} bound at noncatalytic sites [thermolysin (Matthews & Weaver, 1974) and other proteases (Roche & Voordouw, 1977)]. Another class of proteins containing one or more "E-F hand" structural domains with closely related amino acid sequences exhibits high affinity for Ca^{2+} ($K_d \simeq 10^{-6}$–10^{-7} M) at binding sites formed by the junction of two α-helical segments and their connecting loop (Kretsinger, 1976, 1977).

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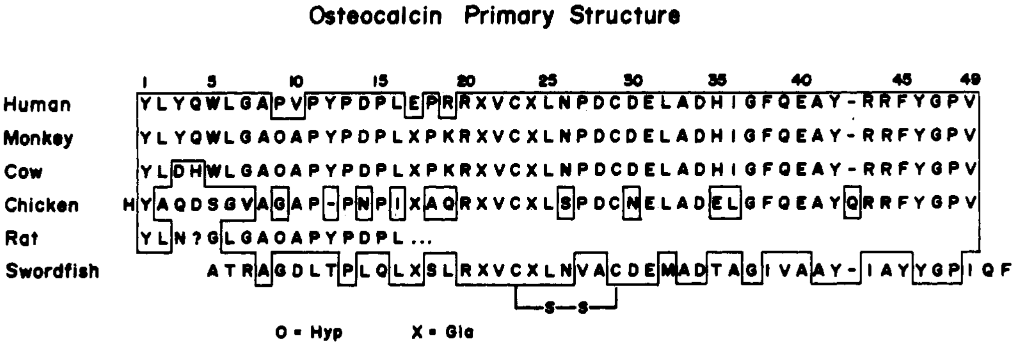

FIGURE 1: Primary structure of osteocalcin in various species. Amino acid sequence data for human (Poser et al., 1980), monkey (Hauschka et al., 1982a), cow (Price et al., 1977), chicken (Carr et al., 1981), rat (Linde et al., 1980), and swordfish (Price et al., 1977) osteocalcins have been aligned for maximum homology and are shown in the one-letter code (IUPAC–IUB, 1969) with Gla represented by X and Hyp by O. For consistency, the numbering of residue positions begins at 1 for the NH_2 terminus of all species except swordfish, which begins at 5. Thus, the COOH-terminal residue is number 49 for human, monkey, and cow osteocalcins, 50 for chicken, and 51 for swordfish. With this scheme, Gla residues are located at positions 17, 21, and 24, and the disulfide bond joins Cys_{23} and Cys_{29} in all species. The boxed-in area encloses all regions of sequence which are identical with the monkey sequence.

These proteins are not known to have enzymatic activity and include muscle calcium binding parvalbumin (Kretsinger, 1977) and troponin C (Potter et al., 1977), the vitamin D induced Ca^{2+}-binding protein of intestinal mucosa (Levine et al., 1977), and the Ca^{2+}-dependent regulatory protein for cyclic nucleotide phosphodiesterase, calmodulin (Klee, 1977; Potter et al., 1977).

A third class of Ca^{2+}-binding proteins, generally of extracellular occurrence, is characterized by the presence of the unique vitamin K dependent amino acid γ-carboxyglutamic acid (Gla).[1] The malonic acid moiety of the Gla side chain apparently participates directly in metal cation binding, either alone or in concert with other Gla and other oxygen-containing ligands (Furie et al., 1979). Prothrombin, with its 10 Gla residues, is the most thoroughly studied of these proteins (Stenflo, 1977). Ca^{2+} mediates a structural transition in prothrombin by a relatively high-affinity interaction ($K_d \sim 2 \times 10^{-4}$ M) of two Ca^{2+} ions with two specific pairs of Gla residues and weaker binding of about six Ca^{2+} ions at other Gla sites (Nelsestuen, 1976; Bloom & Mann, 1978; Marsh et al., 1981; Furie et al., 1979). This binding promotes adoption of the α-helical conformation and facilitates adsorption of prothrombin to acidic phospholipid surfaces (Nelsestuen, 1976; Nelsestuen et al., 1979; Tuhy et al., 1979; Madar et al., 1980).

Osteocalcin[2] is an abundant Ca^{2+}-binding protein of bone matrix (Hauschka et al., 1975; Price et al., 1976) which contains three Gla residues at positions 17, 21, and 24 in its primary structure of 47–51 amino acid residues (M_r 5200–5900, depending on species) (Price et al., 1977; Poser et al., 1980; Linde et al., 1980; Carr et al., 1981; Hauschka et al., 1982a). In addition to its modest affinity ($K_d \sim 8 \times 10^{-4}$ M) for ionic Ca^{2+} (Hauschka & Gallop, 1977), osteocalcin exhibits avid binding to solid calcium phosphate mineral surfaces, particularly where the crystalline phase is hydroxylapatite, $Ca_{10}(PO_4)_6(OH)_2$ (Hauschka et al., 1975; Price et al., 1976; Hauschka & Gallop, 1977; Poser & Price, 1979; Hauschka, 1981a,b). With regard to its calcium mineral adsorption properties, osteocalcin shares certain features with the highly acidic protein statherin (Schlesinger & Hay, 1977; Moreno et al., 1978) and proline-rich proteins of saliva (Bennick et al., 1977), although these other proteins are devoid of Gla.

The propensity of osteocalcin to interact with Ca^{2+}, and the requirement of Gla for this interaction, probably relates to the function of this protein, but as yet no biological role has been unambiguously demonstrated. Osteocalcin appears in developing bone coincident with the onset of mineralization and may be a specific product of cells differentiated with respect to bone formation (Hauschka & Reid, 1978). Osteocalcin is probably synthesized as a higher molecular weight proosteocalcin and subsequently proteolytically processed (Hauschka, 1979a; Lian & Heroux, 1979; Nishimoto & Price, 1980; Hauschka et al., 1982b). Specific radioimmunoassays have revealed circulating levels of osteocalcin in blood plasma (Price & Nishimoto, 1980; Allen et al., 1980) which may be correlated with abnormal states of calcium homeostasis and pathological conditions of bone (Price et al., 1980; Gundberg et al., 1982). Osteocalcin synthesis and distribution are apparently also regulated by vitamin D and its metabolites (Price & Baukol, 1981; Lian et al., 1982). From the variety of properties established for osteocalcin, an important role in Ca^{2+} metabolism may be inferred.

The present paper explores the Ca^{2+}-dependent conformational properties of osteocalcin and demonstrates their consistency with the predicted secondary structure based on the amino acid sequence. A proposed model for osteocalcin is shown to have a unique complementary relationship to the hydroxylapatite crystal lattice. The Ca^{2+}-promoted adsorption of osteocalcin to hydroxylapatite (Hauschka, 1981b) may be understood in terms of the transition to the α-helical structure induced by millimolar levels of free Ca^{2+}.

Experimental Procedures

Purification and Characterization of Osteocalcin. Osteocalcin was extracted and chromatographically purified from powdered bone of chicken, monkey, and rat as described (Carr et al., 1981; Hauschka et al., 1982a,b). Homogeneity was confirmed by invariant integral amino acid composition, Edman end-group analysis, and high-performance liquid chromatography (Hauschka et al., 1982a). Amino acid analysis after acid or alkaline hydrolysis was performed on a Beckman 121M analyzer (Hauschka, 1977). The complete primary structures of the 50-residue chicken osteocalcin (5669 daltons) and the 49-residue monkey osteocalcin (5889 daltons) have been established (Carr et al., 1981; Hauschka et al., 1982a) and are shown in Figure 1.

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; apoosteocalcin, osteocalcin free of multivalent metal cations; RCM-osteocalcin, reduced, carboxymethylated osteocalcin; $3NO_2Tyr$, 3-nitrotyrosine; Gdn·HCl, guanidine hydrochloride; HA, hydroxylapatite; CD, circular dichroism.

[2] Osteocalcin has also been referred to as "bone Gla protein" or "BGP" in the literature (Nishimoto & Price, 1980).

Chemical Modification of Osteocalcin. Apoosteocalcin, free of all bound metal ions, was prepared by dialysis vs. 50 mM HCl at 0 °C for 3 h (Spectrapor membrane, 6000–8000-dalton cutoff), followed by exhaustive dialysis vs. 20 mM Tris-HCl, pH 7.4. Decarboxylation of chicken osteocalcin was achieved by heating the dry acid form of the protein at 110 °C for 280 min in vacuo, reducing the Gla content from 3.04 to 0.59 residues/mol (Hauschka, 1979b). Reduced, carboxymethylated (RCM) osteocalcin was prepared by alkylation with freshly recrystallized iodoacetic acid (Carr et al., 1981). Aliquots of RCM-osteocalcin were decarboxylated as described above. Nitration of Tyr residues in osteocalcin with tetranitromethane (Aldrich) followed the technique of Sokolovsky et al. (1966). The protein concentration was 0.5 mM, with a 7-fold molar ratio of tetranitromethane to Tyr and a Ca^{2+} concentration of 0 or 5 mM. Gel filtration in 0.1 M NH_4HCO_3, employing a 50-cm TSK-2000SW column (Varian) and a Waters liquid chromatograph operated at 1 mL/min (Hauschka et al., 1982a), allowed separation of nitrated osteocalcin from the reaction mixture and was used to monitor the reaction in real time. The extent of nitration was estimated both from the pH-independent 381-nm absorbance at the isosbestic point of 3-nitrotyrosine ($3NO_2Tyr$) in the protein [ϵ = 2200; Sokolovsky et al. (1966)] and by amino acid analysis after acid hydrolysis by using a synthetic $3NO_2Tyr$ standard (Sigma) as described by Giese & Riordan (1975).

Determination of Protein Concentration. Apoosteocalcin (~5 mg/mL) was dialyzed vs. 20 mM Tris-HCl, pH 7.5, centrifuged (10000*g*, 2 min), diluted 10-fold by weight, and sampled quantitatively for amino acid analysis. The absorbance at 276 nm was measured with a Zeiss PMQII spectrophotometer and corrected for scattering by subtraction of the 320-nm absorbance. At 1 mg/mL, the $A_{276} - A_{320}$ values for apoosteocalcin as calibrated by amino acid analysis are as follows: chicken, 0.817; RCM-chicken, 0.761; monkey, 1.41; rat, 1.02. Absorbance was utilized subsequently for all osteocalcin concentration determinations.

Circular Dichroism (CD). CD measurement at 25 °C employed a Cary 61 spectropolarimeter calibrated with *d*-10-camphorsulfonic acid. Molar ellipticity, $[\theta]$, was calculated with a mean residue weight of 11.34 g/dmol for chicken osteocalcin and, following convention, was not corrected for solvent refractive index (Holmquist & Vallee, 1978). CO_2-free buffer (20 mM Tris-HCl, pH 7.4) was boiled and stored under N_2. Stock solutions of divalent metal cations were prepared in the above buffer at 30 mM from metal salts of reagent grade or better. Terbium sulfate (Spex, Metuchen, NJ) was at 2 mM. Stock solutions of apoosteocalcin in 20 mM Tris-HCl, pH 7.4, were diluted appropriately with metal salt solutions and buffer. For each CD measurement in the 185–240-nm range, the 0.202-mm path-length sample cell was filled with freshly diluted 70-μL aliquots of protein at 1–2 mg/mL. CD titrations in the near-UV employed a 10-mm path-length cell. Determination of protein secondary structure from CD parameters involved the curve-fitting procedures of Chen et al. (1972). The estimated precision was ±2% for the α helix and ±5% for the β sheet and random coil. The fraction of the protein existing in the α-helical conformation was calculated from the following relationship: $\%\ \alpha\ \text{helix} = ([\theta]_{222} + 2340)/-30\,300$ as described by Chen et al. (1972).

UV and Fluorescence Titrations. A Cary Model 119 spectrophotometer was used for recording UV difference spectra. Cuvettes containing apoosteocalcin at 0.5 mg/mL in 20 mM Tris-HCl, pH 7.4, were adjusted to zero differential absorbance by using the auto-base-line function, after which Ca^{2+} or pH titration proceeded in one cuvette, with the blank receiving an equal aliquot of buffer. Fluorescence titration was performed at 25 °C on 400-μL samples of osteocalcin in a Perkin-Elmer Model MPF-3 fluorometer. The 280-nm excitation bandwidth was 6 nm, and the intrinsic tyrosine fluorescence emission was filtered (≤290 nm cutoff) and monitored at 308 nm.

Prediction of Protein Conformation. Secondary structure in osteocalcin was predicted from the complete amino acid sequence following the empirical rules of Chou & Fasman (1978). Gla was assumed to have the same structural probability parameters as glutamic acid (Nelsestuen et al., 1979). Electrostatic interactions between the ith residue and its neighbors $i \pm 1, 2, 3, 4$, and 7 were evaluated for disruptive or stabilizing effects on the α-helical structure (Lewis & Bradbury, 1974). Triplets of hydrophobic residues clustered at i, $i + 1$, and $i + 4$ or i, $i + 3$, and $i + 4$ were considered to favor adoption of the α helix (Palau & Puigdomenech, 1974).

Adsorption of ^{125}I-Labeled Osteocalcin to Hydroxylapatite. Hydroxylapatite (HA) of defined surface area (59.56 m^2/g by N_2 adsorption) was kindly provided by Dr. E. C. Moreno of the Forsyth Dental Center, Boston, MA. Pure chicken osteocalcin was iodinated as previously described (Hauschka et al., 1982b), diluted to a specific activity of 4000 cpm/μg, and dialyzed against the adsorption buffer (150 mM NaCl–20 mM Tris-HCl, pH 7.6). Preequilibration of HA for 20 h at 4 °C (0.1–20 mg of HA/350 μL of buffer in 1.5-mL plastic centrifuge tubes) was followed by addition of 150 μL of buffer containing ^{125}I-labeled osteocalcin (30 μg/mL final concentration), Ca^{2+}, and other test substances. Adsorption at 4 °C for 20 h with constant shaking was terminated by centrifugation and analysis of supernatant samples with a Micromedics γ counter. Data were analyzed by computer according to the Langmuir adsorption isotherm model of Moreno et al. (1978).

Results

Osteocalcin Exists Primarily in the Random Coil Conformation in the Absence of Ca^{2+}. The CD spectrum of metal-free osteocalcin (apoosteocalcin) is rather featureless with a trough at 204 nm (Figure 2). The best fit (Chen et al., 1972) to this curve is 8% α helix, 14% β sheet, and 78% random coil, which translates to four or five residues in the α-helical conformation. Electrostatic repulsion looms as the major factor in determination of this structure. Previously, apoosteocalcin was observed to have aberrant behavior on gel filtration columns, indicative of an extended chain conformation; polyelectrolyte effects on the pH dependence of Ca^{2+} binding have also been observed (Hauschka & Gallop, 1977). The net charge of chicken osteocalcin is −9, assuming uncharged histidine at pH 7.4. Decarboxylation of the Gla residues reduces the net charge to between −6 and −7, allowing the α-helical content to approach 18% or about nine residues in the α helix (Figure 2, Table I). Increased electrostatic shielding provided by 1.5 M NaCl allows apoosteocalcin to adopt 15% α-helical structure, while 4 M guanidine hydrochloride (Gdn·HCl) abolishes all α helix (Table I). Reduction and carboxymethylation of the disulfide bridge increases the net charge to −11, and without this covalent restraint, no α helix is found (Table I).

Ca^{2+} Induces α-Helical Structure in Osteocalcin, and Gla Residues Are Required for This Transition. CD spectra reveal that a major conformational change occurs in osteocalcin when Ca^{2+} ions are bound. Deep troughs develop at 222 and 207 nm, and a positive CD peak occurs at 196 nm in 5 mM Ca^{2+} (Figure 2). This spectrum is compatible with a secondary

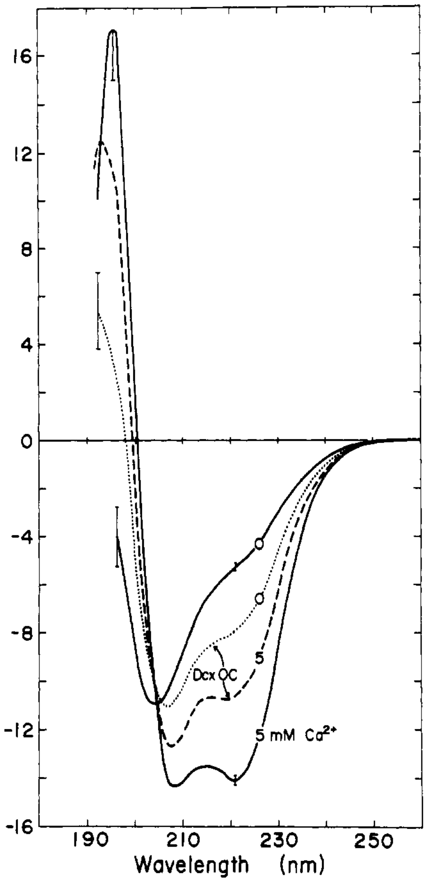

FIGURE 2: Circular dichroism (CD) spectra of native and decarboxylated chicken osteocalcin in the presence and absence of 5 mM Ca^{2+}. Metal-free native apoosteocalcin shifts dramatically from a predominantly random coil structure (—, 0) to an α-helical conformation when Ca^{2+} is added (—, 5 mM Ca^{2+}). Decarboxylated apoosteocalcin shows slightly more α helix in the absence of Ca^{2+} (···, 0), but only one-fourth as large a conformational shift is induced by Ca^{2+} (- - -, 5). The CD spectra were invariant over time. Protein concentration was 1.7 mg/mL in 150 mM NaCl-20 mM Tris-HCl, pH 7.4. Error bars at 196 and 220 nm show the actual noise level observed during multiple scans at 3 nm/min with an instrument time constant of 10 s.

Table I: Calcium-Induced α Helix in Osteocalcin

osteocalcin	[Ca^{2+}] (mM)	$[\theta]_{222}$ (deg·cm^2/dmol)	% α helix^a
native	0	−4761	8
	5	−13990	38
native + 1.5 M NaCl	0	−6900	15
	5	−12820	35
decarboxylated	0	−7700	18
	5	−10370	26
RCM	0	−1700	−2
	5	−3100	3
RCM-decarboxylated	0	−1970	−1
	5	−1950	−1
native + 4 M Gdn·HCl	0	−1080	−4
native + 1 M Gdn·HCl	5	−4600	7

[a] % α helix = ($[\theta_{222}$ + 2340)/−30 300.

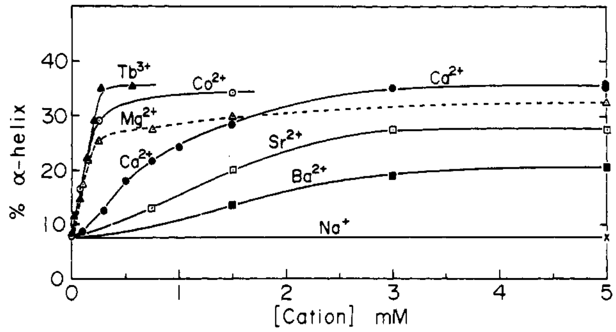

FIGURE 3: Metal cation induced α-helical structure in chicken osteocalcin. The content of α helix was calculated from $[\theta]_{222}$ (Chen et al., 1972). Each point represents CD data from a separate 70-μL aliquot of apoosteocalcin after cation addition. Scans from 250 to 185 nm were made immediately after mixing, and [θ] values were invariant over time. The protein concentration was 1.7 mg/mL in 150 mM NaCl-20 mM Tris-HCl, pH 7.4, except for the Tb^{3+} curve which was plotted from Figure 4. Midpoints for induction of α helix occur at 0.11 mM Co^{2+} (○), 0.14 mM Tb^{3+} (▲), 0.14 mM Mg^{2+} (△), 0.75 mM Ca^{2+} (●), 1.7 mM Sr^{2+} (□), and 7.0 mM Ba^{2+} (■).

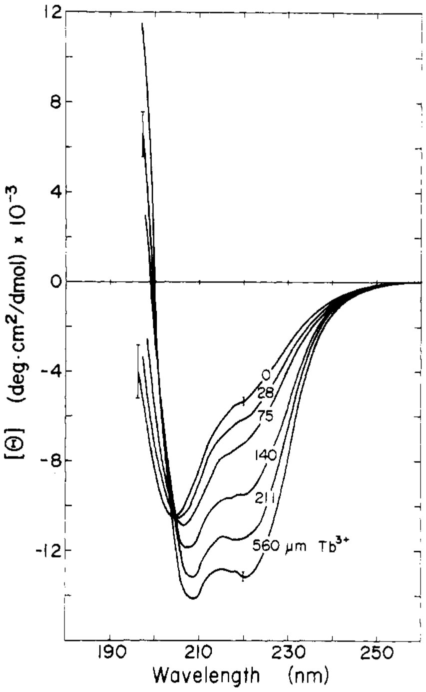

FIGURE 4: CD spectra of chicken apoosteocalcin in the presence of increasing amounts of Tb^{3+}. Conditions were identical with those of Figure 2, except that the solvent was 20 mM Tris-HCl, pH 7.4. The noncooperative transition to the α-helical conformation exhibits a midpoint at 0.14 mM Tb^{3+}.

structure consisting of 38% α helix, 8% β sheet, and 45% coil. Thus, in the presence of Ca^{2+}, some 19 residues are in the α-helical conformation. A similar magnitude of Ca^{2+}-induced α-helical structure is observed for monkey and rat osteocalcins by CD (data not shown). The role of the three Gla residues in binding Ca^{2+} and inducing this conformational change is clearly shown by the decarboxylated chicken protein acquiring only 8% additional α helix in the presence of Ca^{2+} (Figure 2, Table I). When electrostatic effects are corrected for, this change is only one-fourth the magnitude of that induced by Ca^{2+} in the native Gla-containing osteocalcin. It is noteworthy that 1.5 M NaCl does not prevent induction of the α helix by 5 mM Ca^{2+}, while 1 M Gdn·HCl does, and that an intact disulfide bond is required for the conformational change (Table I).

The induction of the α helix has been studied by titration of apoosteocalcin with Ca^{2+} and other metal cations (Figures 3 and 4). For Ca^{2+}, the transition midpoint for the α helix occurs at 0.75 mM, virtually identical with the K_d of 0.8 mM observed for the equilibrium dialysis binding of Ca^{2+} ions (Hauschka & Gallop, 1977). Both processes are noncooperative with a Hill coefficient of $n = 1$. Other metals induce α-helical structural transitions in osteocalcin with midpoint

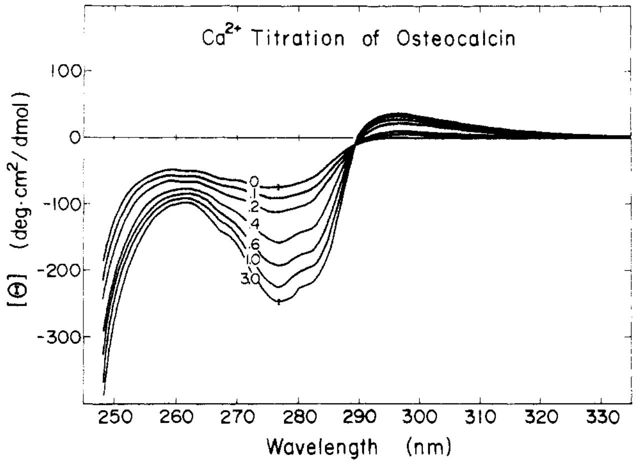

FIGURE 5: Near-ultraviolet CD spectra of chicken osteocalcin at increasing concentrations of Ca^{2+}. Apoosteocalcin (1.6 mg/mL in 150 mM NaCl–20 mM Tris-HCl, pH 7.4) was titrated in a 1-cm path cell and scanned repeatedly after each addition of Ca^{2+}; spectra were invariant over time. The spectrum at 5 mM Ca^{2+} was superimposable with that at 3 mM.

concentrations increasing in the order Co^{2+} < Tb^{3+}, Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+} (Figure 3). Progressive adoption of the α-helical conformation by apoosteocalcin in the presence of incremental amounts of Tb^{3+}, a lanthanide series analogue of Ca^{2+} (Brittain et al., 1976), is shown in Figure 4. Both Tb^{3+} and La^{3+} were previously shown to competitively displace Ca^{2+} from its binding sites in osteocalcin (Hauschka & Gallop, 1977).

Near-Ultraviolet CD Shows Ca^{2+}-Dependent Alteration of Tyrosine Environments. Apoosteocalcin exhibits a weak negative Cotton effect in the Tyr absorption band ($[\theta]_{277}$ = −76 ± 4 deg·cm^2/dmol) when examined by CD (Figure 5). As Ca^{2+} is added to the protein, this trough deepens dramatically ($[\theta]_{277}$ = −250 ± 4 deg·cm^2/dmol) and develops shoulders at 282 and 267 nm. This is strong evidence that the environments of one or more Tyr residues and possibly Phe are altered concomitant with the adoption of the α-helical conformation (Horwitz et al., 1970). Tyr residues are located at positions 2, 42, and 47 in chicken osteocalcin, and, as discussed below, Tyr$_{42}$ is at the terminus of an α-helical domain and borders one of the Ca^{2+} binding sites. Another chromophore that could contribute to the CD spectrum in this region is the disulfide bond joining Cys$_{23}$ and Cys$_{29}$ (Horwitz et al., 1970; Beychok, 1967). However, the weak negative CD band between 250 and 320 nm characteristic of disulfides such as L-cystine (Beychok, 1967) would probably not account for more than −40 deg·cm^2/dmol or about 15% of the total CD in the presence of Ca^{2+}. In addition, a broad *positive* Cotton effect at 295 nm ($[\theta]$ = 36 ± 4 deg·cm^2/dmol) appears in the presence of Ca^{2+} (Figure 5). This change is half complete at 0.41 mM Ca^{2+}. Appearance of the positive CD signal at 295 nm suggests that Ca^{2+} may induce phenolate ion formation well below the normal pK_a of 9.8 for Tyr. The phenolate ion of Tyr absorbs maximally at 295 nm and would be stabilized by a propinquitous cation such as Ca^{2+}.

Ca^{2+} Induces Ultraviolet Absorption Difference Spectrum. Figure 6 shows the perturbation of the protein absorption spectrum caused by the binding of Ca^{2+}. The appearance of positive double peaks at 287 nm ($\Delta\epsilon^{M}$ = 380, based on the molar concentration of osteocalcin) and 279 nm ($\Delta\epsilon^{M}$ = 180), as well as at 237 nm ($\Delta\epsilon^{M}$ = 1220), is characteristic of a red shift of the Tyr absorption band (Herskovitz, 1967) and usually signifies decreased exposure of Tyr residues to the surrounding polar solvent (Horwitz et al., 1970). Interpretation of the difference spectrum of chicken osteocalcin is simplified by the absence of tryptophan in this protein. Fine structure in the Phe absorption band [$\Delta\epsilon^{M}_{267}$ = −71, $\Delta\epsilon^{M}_{264}$ = −84, and $\Delta\epsilon^{M}_{258}$ = −75 (Figure 6)] indicates that the two Phe residues at positions 38 and 46 may also be perturbed by the Ca^{2+}-induced conformational transition (Horwitz et al., 1970). Both Phe residues are apparently part of a hydrophobic cluster which includes Ile$_{16}$, Pro$_{12}$, and Tyr$_{42}$ in the Ca^{2+}–osteocalcin complex (see below).

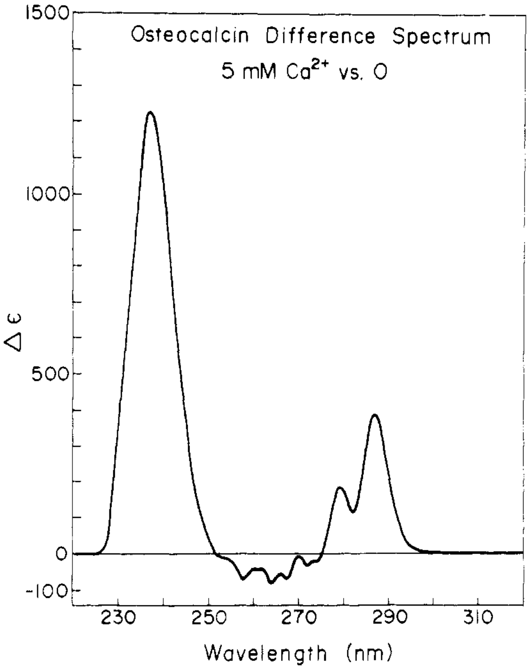

FIGURE 6: Ultraviolet difference spectrum of chicken osteocalcin caused by the presence of 5 mM Ca^{2+}. The zero base-line was established with apoosteocalcin in both sample and reference cuvettes at 0.357 mg/mL in 150 mM NaCl–20 mM Tris-HCl, pH 7.4. Addition of Ca^{2+} to 5 mM final concentration in the sample, with parallel addition of buffer to the reference cuvette, caused an instantaneous red shift of the Tyr absorption bands (positive peaks at 287, 279, and 237 nm) and perturbation of Phe (negative peaks at 267, 264, and 258 nm).

Intrinsic Tyr Fluorescence in Osteocalcin Is Decreased by Ca^{2+}. Apoosteocalcin exhibits a characteristic Tyr fluorescence spectrum with an uncorrected emission maximum of 308 nm. Upon addition of Ca^{2+}, the fluorescence intensity decreases monotonically to 60% of its original value. This change is complete by 3 mM final [Ca^{2+}] and is noncooperative. As in all the interactions of osteocalcin with metal cations, decreased ionic strength increases the affinity of the protein for Ca^{2+} (Hauschka & Gallop, 1977). The midpoint for completion of the fluorescence decrease is 0.55 mM Ca^{2+} at physiological salt concentrations (150 mM NaCl–20 mM Tris-HCl, pH 7.4), while at low ionic strength (20 mM Tris-HCl, pH 7.4) only 0.15 mM Ca^{2+} is required. Interpretation of these fluorescence changes in terms of polarity of the Tyr environment or partial phenolic ionization of Tyr is not warranted by the available data, yet the conformational transition clearly affects Tyr residues.

Reactivity of Tyr to Tetranitromethane Is Decreased by Ca^{2+}. Nitration of Tyr residues (Sokolovsky et al., 1966) was explored as a possible means to identify the individual residue(s) in chicken osteocalcin which were most strongly perturbed by the Ca^{2+} induction of the α-helical conformation. In the absence of Ca^{2+}, 3NO$_2$Tyr formed rapidly and quantitatively at all three Tyr positions, based on amino acid analysis of tryptic fragments and molar extinction of the ni-

Table II: Predicted Secondary Structure in Osteocalcin[a]

	α helix		β sheet		β turn	
species	residues[b]	P_α	residues	P_β	residues	$[p_t] \times 10^4$
human	$Pro_{18} \rightarrow Leu_{25}$ $Asp_{30} \rightarrow Ala_{41}$ (41)	1.12 4.38	$Tyr_1 \rightarrow Ala_8$ $Tyr_{42} \rightarrow Gly_{47}$ (29)	3.43 1.93	$Tyr_{12} \rightarrow Pro_{15}$ $Asn_{26} \rightarrow Cys_{29}$	3.00 11.10
monkey	$Pro_{18} \rightarrow Leu_{25}$ $Asp_{30} \rightarrow Ala_{41}$ (41)	1.33 4.38	$Tyr_1 \rightarrow Ala_8$ $Tyr_{42} \rightarrow Gly_{47}$ (29)	3.43 1.93	$Tyr_{12} \rightarrow Pro_{15}$ $Asn_{26} \rightarrow Cys_{29}$	3.00 11.10
cow	$Pro_{18} \rightarrow Leu_{25}$ $Asp_{30} \rightarrow Ala_{41}$ (41)	1.33 4.38	$Tyr_1 \rightarrow Gly_7$ $Tyr_{42} \rightarrow Gly_{47}$ (27)	1.20 1.93	$Tyr_{12} \rightarrow Pro_{15}$ $Asn_{26} \rightarrow Cys_{29}$	3.00 11.10
chicken	$Ile_{16} \rightarrow Leu_{25}$ $Asn_{30} \rightarrow Ala_{41}$ (44)	5.16 4.92	$Tyr_{42} \rightarrow Gly_{48}$ (14)	2.13	$Asp_5 \rightarrow Val_8$ $Pro_{12} \rightarrow Pro_{15}$ $Ser_{26} \rightarrow Cys_{29}$	2.06 3.99 8.28
swordfish	$Pro_{13} \rightarrow Ala_{33}$ (45)	5.80	$Ile_{38} \rightarrow Phe_{51}$ (30)	7.92	$Arg_7 \rightarrow Asp_{10}$ $Asp_{34} \rightarrow Gly_{37}$	0.82 0.84

[a] Probability parameters were calculated according to the definitions of Chou & Fasman (1978). P_α, P_β, and $[p_t]$ are indicated for each structural domain. The percentage of the osteocalcin molecule expected in the α-helical and β-sheet conformations is shown in parentheses. [b] Sequence positions follow the convention in Figure 1.

trated protein [ϵ_{381} = 6420 vs. 6600 expected (Sokolovsky et al., 1966)]. A 2–4-fold reduction in the rate of nitration was caused by the presence of 5 mM Ca^{2+}, strongly indicating that the α-helical transition reduced the availability of Tyr for reaction with tetranitromethane. However, it was also apparent that even in the α-helical conformation, the same final extent of Tyr nitration was eventually attained. The reduced nitration rate which accompanies the binding of Ca^{2+} to osteocalcin as well as to troponin C (McCubbin et al., 1979) parallels the burying of Tyr in less polar environments (red shift), whereas the increased exposure of Tyr_{138} in calmodulin to a more polar environment (blue shift) is reflected by its increased reactivity to tetranitromethane (Richman & Klee, 1978).

A final observation which corroborates the solvent exposure of all three Tyr residues in apoosteocalcin is the normal alkaline titration properties of these residues (pK_a ~10.0 ± 0.2). Also, the absorption difference peaks at 243 and 295 nm which accompany formation of the phenolate ion in alkali have a ratio of only 4.0 over the entire titration, making it particularly unlikely that buried Tyr chromophores have been suddenly exposed to solvent during the titration (Mihalyi, 1968).

Predicted Secondary Structure in Osteocalcin. Osteocalcins of all species share extensive amino acid sequence identity, arguing for functional preservation throughout evolution (Figure 1). Thus, there are common features in the secondary structure predicted by the method of Chou & Fasman (1978). The α-helical conformation is predicted for 41–45% of the total osteocalcin molecule, depending on the species (Table II), in reasonable agreement with the 38% observed by CD in the presence of Ca^{2+}. In most species, two α-helical domains are predicted: (1) the "Gla helix" contains two or three of the Gla residues and extends from position 16 (chicken) or 18 (human, monkey, cow) to position 25; (2) the "Asp-Glu helix" comprising sequence positions 30–41 contains four anionic side chains. In swordfish, the most divergent osteocalcin for which sequence information is available (Price et al., 1977), a single long helix probably occupies positions 13–33. β-sheet structure is predicted at the COOH-terminal regions of all osteocalcins, with a small segment also expected at the NH_2 terminus of the mammalian proteins (Table II).

Tertiary folding of globular proteins is accomplished in part through β turns (Chou & Fasman, 1974) which consist of four amino acid residues and permit nearly 180° reversal of polypeptide chain direction. β turns may connect α helices, β sheets, or random coil segments (Crawford et al., 1973), and their positions are highly conserved in proteins such as the immunoglobulins, where β turns are believed to connect the hypervariable regions (Bunting et al., 1972). In osteocalcin, predicted β turns also appear to serve important functions in back-folding of the molecule. Chicken and mammalian osteocalcins share highly probable β turns at residues 26–29 ($[p_t]$ = 8.3 × 10^{-4} for chicken; 11.1 × 10^{-4} for mammalian; Figure 7 and Table II). The occurrence of a disulfide bond in this same region (Cys_{23}–Cys_{29}) may further stabilize the β turn and force the two neighboring α-helical segments into close antiparallel approximation.

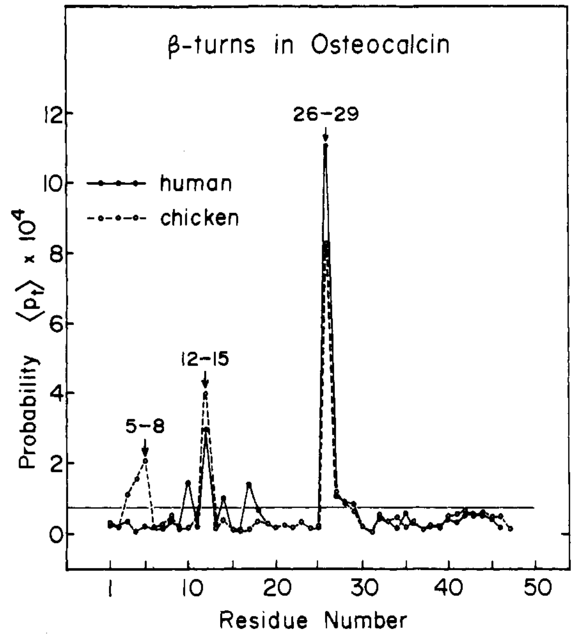

FIGURE 7: Probability of β turns in human (●) and chicken (○) osteocalcin. Calculation of probability followed the method of Chou & Fasman (1978), and $[p_t]$ values are plotted for β turns of four residues commencing at the *i*th position in the sequence. The horizontal line at $[p_t]$ = 0.75 × 10^{-4} represents a cut-off point for selection of β turns as recommended by Chou & Fasman (1978).

High anionic charge density in the predicted α-helical segments necessitated analysis of electrostatic interaction

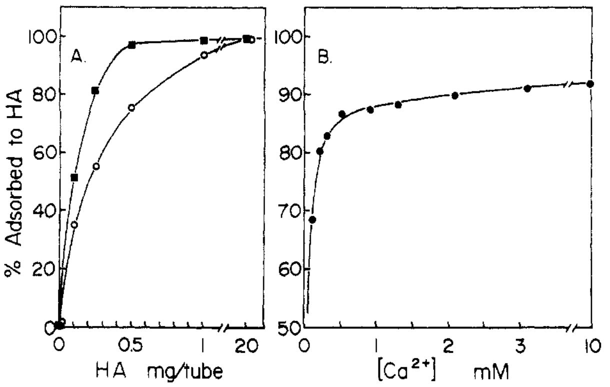

FIGURE 8: Ca^{2+}-dependent adsorption of chicken osteocalcin to synthetic hydroxylapatite (HA). Binding of ^{125}I-labeled osteocalcin to powdered, crystalline HA of defined specific surface area (59.56 m^2/g) was measured after a 20-h equilibration at 4 °C following the general procedures of Moreno et al. (1978). (A) Adsorption expressed as the fraction of bindable protein associated with the HA pellet at 0.1 mM Ca^{2+} (○) and 6 mM Ca^{2+} (■). (B) $[Ca^{2+}]$ dependence of adsorption to a constant amount of HA, 400 μg (●).

energy (Lewis & Bradbury, 1974). In the "Asp-Glu helix", three repulsive interactions between Glu_{31}, Asp_{34}, and Glu_{35} exert a helix-breaking effect. In the "Gla helix", because of the double negative charges on Gla side chains and their spacing at i, $i + 4$, and $i + 7$, 12 repulsive interactions exist. This destabilizing influence should be largely abolished by the binding of two or three Ca^{2+} ions or other cations. Significantly, this repulsion is also partially offset by the critically placed positive charge at Arg_{20} which allows six attractive interactions with the three Gla residues. The invariant occurrence of Arg_{20} in all osteocalcins as well as an additional positive charge, Lys_{19} or Arg_{19} in some species (Figure 1), hints at the importance of this internal charge neutralization which may be a prerequisite for nucleation of the α-helical conformation in the "Gla helix". Hydrophobic stabilization within predicted helical segments may also balance the destabilizing influence of electrostatic repulsion. The arrangements of Ala_{18}-Val_{22}-Leu_{25} and Leu_{32}-Ala_{33}-Leu_{36}-Phe_{38} are favorable to α-helix formation (Palau & Puigdomenech, 1974).

A striking feature of the predicted α-helical domains in osteocalcin is the regular spacing of both anionic and hydrophobic amino acid side chains at intervals of three or four residues (e.g., Gla_{17}, Gla_{21}, Gla_{24}). Because the pitch of the Pauling-Corey α helix is 3.6 residues/turn, one face of the helical surface is distinctly anionic, while the backside is hydrophobic. Such polarization of α-helix surfaces is a common occurrence in proteins (Chou & Fasman, 1978), and these so-called amphipathic helices are usually oriented with the polar face projecting outward into the aqueous solvent environment. Furthermore, the α-helix periodicity of 5.4 Å/turn forces the Gla residues into register such that their side chains are most probably ~5.4 Å apart. This is apparently a highly significant structural feature of osteocalcin which may govern its interaction with hydroxylapatite and other solid phases of calcium phosphate (see below, and Figure 9).

Ca^{2+}-Dependent Adsorption of Osteocalcin to Hydroxylapatite (HA). Helical conformation is important for the adsorption of osteocalcin to HA. The conformational transition induced by 5 mM Ca^{2+} greatly increases the affinity of the protein for the mineral surface (Figure 8A). The affinity constant at 4 °C based on the Langmuir adsorption isotherm model of Moreno et al. (1978) is ~12 000 mL/μmol in the presence of Ca^{2+}, compared to only ~1100 mL/μmol for the apoprotein. It is important to note that since the mineral phase is of constant specific surface area, a parameter which is not known to be affected by low concentrations of free Ca^{2+}, the changes in the affinity constant must be due to the conformational transition in osteocalcin. Titration of this transition, and the attendant increase in affinity, is shown for Ca^{2+} in Figure 8B. The Ca^{2+} concentration dependence of binding to HA is rather similar to the α-helix induction curve in Figure 3. Possible physiological significance of these effects is discussed below.

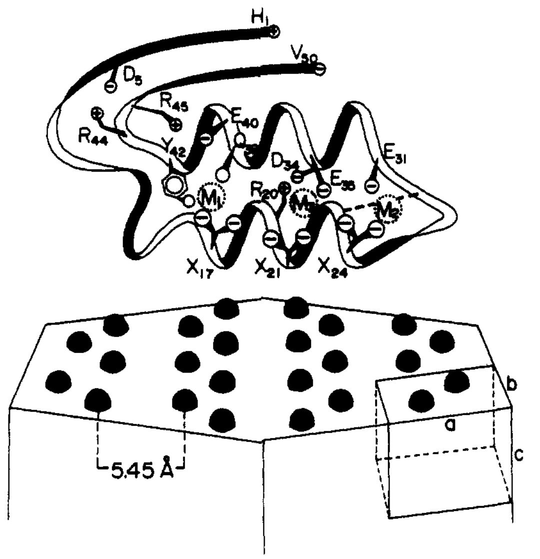

FIGURE 9: Model for the Ca^{2+}-induced structure of osteocalcin showing its probable mode of interaction with Ca^{2+} in the hydroxylapatite crystal lattice. The 50 amino acid polypeptide backbone of chicken osteocalcin is represented by a ribbon drawn to the same scale as indicated for the crystal. Important side chains are labeled in the one-letter code with a subscript corresponding to the sequence position [D, Asp; E, Glu; H, His; Q, Gln; R, Arg; V, Val; X, Gla; Y, Tyr]. Formal electronic charges are indicated as they would exist in the protein at pH 7–8. Progressing from the NH_2 terminus (H_1), β turns occur at positions 5 → 8 and 12 → 15; the "Gla helix" occupies residues 16 → 25; a β turn at 26 → 29 is stabilized by the Cys_{23}–Cys_{29} disulfide bond (− − −); the "Asp-Glu helix" extends from 30 to 41; finally, the COOH terminus ending at V_{50}, with its β-sheet structure from 42 to 48, lies in propinquity to the NH_2 terminus and probably covers the hydrophobic backsides of the helices. The probable metal binding sites are labeled M_1, M_2, and M_3, and some possible coordinating oxygen-containing side chains are indicated. Note that all three Gla residues project in the same direction away from one face of the "Gla helix". The periodic spacing of Gla residues (~5.4 Å) and opposing anionic side chains of the "Asp-Glu helix" is highly complementary to the 5.45-Å interatomic distance shown for the hexagonally disposed Ca_I in the xy plane (001) of the hydroxylapatite lattice. This may explain the tight adsorption of osteocalcin to such surfaces. Crystallographic coordinates and unit-cell dimensions for hydroxylapatite are those of Kay et al. (1964).

Discussion

Ca^{2+}-Dependent Protein Structure. The conformational transition induced in osteocalcin by Ca^{2+} is amply documented by CD (Figures 2–5), UV difference spectrum (Figure 6), fluorescence, and HA binding affinity (Figure 8). The 30% net increase in α helix which occurs when Ca^{2+} is bound means that about one-third of the entire protein is suddenly able to adopt this structure. The prediction that 22 residues should be α helical in chicken osteocalcin (Table II) is in excellent agreement with the 19 found to be in this conformation in the presence of Ca^{2+} (38% α helix, Table I). The reliability of the predictive method is especially good for small proteins where long-range interactions are minimal (Chou & Fasman, 1978); thus, it is most probable that the α helix observed by CD occurs in the specified regions (Table II) as diagrammed in Figure 9. Clearly, there is an important permissive effect

of Ca^{2+} involving charge neutralization before the full α-helical potential of osteocalcin can be realized. The location of the four- or five-residue α-helical segment in the metal-free protein is uncertain, but it may surround Arg_{20} because of favorable electrostatic effects in this locus.

Osteocalcin is not the only Ca^{2+}-binding protein which responds to $[Ca^{2+}]$ with a conformational transition. Calmodulin (Klee, 1977), troponin C (Murray & Kay, 1972), and prothrombin (Bloom & Mann, 1978) also exhibit such changes; however, the fraction of the molecule involved ranges from 5 to 22%, which is smaller than the 30% for osteocalcin. An interesting *slow* transition also occurs in prothrombin and possibly involves cis–trans isomerization of particular prolyl peptide bonds which, once stabilized by Ca^{2+}, allows specific interaction with phospholipid (Nelsestuen, 1976; Marsh et al., 1979); slow transitions of this type have not been found in osteocalcin.

Model for Osteocalcin Structure. Combined chemical, spectral, and predictive investigations of osteocalcin structure have yielded the model shown in Figure 9. A CPK space-filling model was constructed corresponding to residues 1 → 50 for chicken osteocalcin, with precise assembly of α-helical and β-sheet structures as previously described (Hauschka & Segal, 1966). This model, which is compatible with all available data and the predictions in Table II, consists of two antiparallel α-helical domains, the "Gla helix" (residues 16 → 25) and the "Asp-Glu helix" (residues 30 → 41), connected by a peptide segment containing a β turn (residues 26 → 29) and stabilized by the Cys_{23}–Cys_{29} disulfide bond. Other β turns occur at positions 5 → 8 and 12 → 15, with β-sheet structure in the COOH terminus from 42 to 48 (Figure 9). Additional constraints derive from immunochemical studies which indicate propinquity of the NH_2 and COOH termini of osteocalcin (Hauschka et al., 1982b). Perhaps the most important feature of the model is that all three Gla residues project from one surface of the "Gla helix". This periodic spacing of Gla side chains, in combination with opposing anionic side chains of the "Asp-Glu helix", apparently dictates the binding parameters for metal cations and calcium phosphate mineral surfaces.

Another interesting structural feature of osteocalcin is the opportunity for formation of stabilizing hydrophobic clusters. A major cluster evident in the space-filling model involves Pro_{12}, Ile_{16}, Phe_{38}, Tyr_{42}, and Phe_{46}. Ile_{16} occurs at one end of the "Gla helix" and may anchor it to the hydrophobic cluster. The probable significance of this cluster is underscored by the fact that the hydrophobic character of all of its component residues has been strongly conserved during evolution (Figure 1). In the "E-F hand" family of Ca^{2+}-binding proteins, there exists a single Ile residue which is almost invariant among species and which appears to hold the Ca^{2+}-binding loop to the hydrophobic core of the molecule (Kretsinger, 1977).

Metal Binding Sites. Two metal cation binding sites, M_1 and M_2, are clearly evident in the space-filling model of osteocalcin, and a third, M_3, probably depends on the disposition of the Arg_{20} side chain (Figure 9). Previous studies of Ca^{2+} binding to chicken osteocalcin by equilibrium dialysis revealed two relatively high affinity sites with $K_d = 0.8$ mM and two or three weaker sites with $K_d \simeq 3$ mM (Hauschka & Gallop, 1977). In cow osteocalcin, there are three Ca^{2+} binding sites with average $K_d \simeq 3$ mM (Price et al., 1977). Ca^{2+} coordination in proteins generally involves six to eight oxygen-containing ligands, a role dominated by carboxyl groups, peptide carbonyl groups, and water (Kretsinger, 1976). In M_1 (Figure 9), Gla_{17} can provide a carboxyl group, with peptide and side-chain carbonyls from Gln_{39}. Interestingly, Tyr_{42} is juxtaposed to M_1 in such a way that its environment would most likely be perturbed by occupation of this site by Ca^{2+}. Spectral studies (Figures 5 and 6) prove that at least one Tyr residue is sensitive to metal binding, but the exact assignment has not yet been made. The M_2 site provides possible coordination through carboxyl groups of Gla_{24} and Glu_{31}, as well as the peptide carbonyl of Gla_{24}. The availability of M_3 for occupancy probably depends on whether Arg_{20} is buried, forming a salt bridge with Asp_{34}, or is occluding the site by interaction with Gla_{17}, Gla_{21}, or Glu_{35}. Arg_{20} may also act to stabilize H_2O as an additional ligand for Ca^{2+}, as it does in concanavalin A (Becker et al., 1975). The predicted secondary and tertiary structures for cow osteocalcin are virtually identical with those for chicken osteocalcin (Table II, Figure 9), and it is likely that all three sites M_1, M_2, and M_3 are functional in the cow protein with the additional positive charge at Lys_{19}, possibly explaining the reduced affinity for Ca^{2+} compared to chicken osteocalcin.

Furie et al. (1979) and Marsh et al. (1981) have described the geometry of lanthanide-series cation coordination by Gla for both the free amino acid and the Gla residues in prothrombin. Apparently, the two carboxyl groups of a single Gla can occupy at most two of the coordination sites for these analogues of Ca^{2+}. In other cases, a single carboxyl group of Asp acts as a ligand for two different cations [e.g., Asp_{10} coordinates both Ca^{2+} and Mn^{2+} in adjacent sites on concanavalin A (Becker et al., 1975)]. The relevance of these observations to osteocalcin is that even when sites M_1 and M_2 are occupied, single carboxyl groups of Gla_{17} and Gla_{24} and possibly both carboxyl groups of Gla_{21} could still be free for interaction with additional cations, including Ca^{2+} in the surface of the HA lattice (Figure 9).

Role of Gla in the Binding of Ca^{2+}. The importance of Gla residues in prothrombin for the binding of Ca^{2+}, adsorption to phospholipid, and factor Xa mediated activation has been clearly demonstrated (Stenflo, 1977; Nelsestuen et al., 1979; Furie et al., 1979). Similar importance may now be attributed to Gla in osteocalcin. Thermally decarboxylated osteocalcin undergoes only one-fourth as much shift to the α helix upon adding Ca^{2+} as does native osteocalcin (Figure 2). At the same time, the normal appearance of the CD spectrum for decarboxylated protein indicates that the decarboxylation process did not drastically alter the protein (Tuhy et al., 1979). Poser & Price (1979) have previously shown that osteocalcin loses affinity for HA after decarboxylation. Also, they demonstrated a protective effect whereby HA prevents Gla decarboxylation in adsorbed osteocalcin. We have demonstrated through biodistribution studies of ^{125}I-labeled osteocalcin in rats that decarboxylated osteocalcin is less than one-third as effectively adsorbed to bone mineral in vivo and that it is cleared from the circulation up to 10 times faster (Hauschka, 1981c). Taken in concert, these results indicate the intrinsic involvement of Gla residues in the interactions of osteocalcin with Ca^{2+}, both in solution and in solid mineral phases.

Interaction of Osteocalcin with Hydroxylapatite (HA). The known affinity of osteocalcin for HA surfaces (Figure 8) and the structural model for osteocalcin with its periodically spaced (~5.4 Å) Gla residues prompted the depiction in Figure 9. Of the inter-Ca^{2+} distances observed in the HA lattice (Kay et al., 1964), only two are compatible with the predicted spacing of Gla residues and Ca^{2+} binding sites in osteocalcin: Ca_I–Ca_I (5.45 Å, *xy* plane); Ca_{II}–Ca_{II} (5.84 Å, *xz* and *yz* planes). Other calculated spacings are 3.44, 4.06 ± 0.14, 6.33, 6.88, and 9.43 Å. Possibly, preferential interaction occurs with the xy surfaces (001 plane) of HA crystals (Figure 9). In bone, the unit-cell c axis is parallel to the 400-Å-long axis of the lath-shaped microcrystals (Glimcher, 1959). Thus, the 5.45-Å Ca_I hexagonal array is accessible only at crystal ends. Interestingly, there is about one molecule of osteocalcin for each HA microcrystal in bone.

It is apparent from the elegant complementarity to HA that the α-helical conformer of osteocalcin binds more tightly (Figures 8 and 9), but questions arise regarding the Ca^{2+} ions occupying the metal binding sites. Protein-bound Ca^{2+} may be available for interaction with Ca^{2+} vacancy positions in the crystal surface, depending on the details of the coordination. Alternately, lattice Ca^{2+} may occupy the metal binding sites on the protein surface. An intriguing possibility is that Arg_{20} and the basic residue at position 19 in some species may also interact directly with phosphate ions in the HA lattice. Specific binding of phosphate esters by certain enzymes involves Arg–phosphate interaction (Riordan, 1979). Thus, in osteocalcin, the highly conserved charge alternation in the "Gla helix" (= + = = or = + + = =) might be a critical factor in HA adsorption.

Physiological Significance of the Ca^{2+}-Induced Helical Transition. According to Kretsinger (1976, 1977), the binding of Ca^{2+} to proteins plays an informational role and provides for biological regulation through ion transport, protein conformational change, and enzyme activation. These effects may operate over a wide range of $[Ca^{2+}]$. Typical *intracellular* Ca^{2+}-binding proteins (troponin C, muscle parvalbumin, calmodulin, and vitamin D dependent protein of intestinal mucosa) exhibit extremely high affinities for Ca^{2+} with K_d values of 10^{-6}–10^{-7} M, as these proteins are located in cytosolic environments where free $[Ca^{2+}]$ hovers around 10^{-7} M (Kretsinger, 1977). *Extracellular* (plasma) proteins such as prothrombin and osteocalcin are typically bathed by millimolar levels of free Ca^{2+} which is in line with the observed K_d range of 10^{-3}–10^{-4} M for Ca^{2+}.

An important feature of osteocalcin is its occurrence in blood (Price & Nishimoto, 1980; Allen et al., 1980). Measurement of serum osteocalcin in a variety of situations indicates that only 10^{-4}–10^{-5} of the total body osteocalcin is freely circulating. It is unclear whether the circulating pool provides information of a hormonal nature or is merely a consequence of the adsorption equilibrium with bone mineral. Correlations of elevated or depressed serum osteocalcin levels have been made with certain pathological states of bone and abnormalities of Ca^{2+} metabolism (Price et al., 1980; Gundberg et al., 1982). Interpretation of these changes must be based on a solid understanding of the physicochemical parameters governing osteocalcin–mineral interaction. We have demonstrated that free $[Ca^{2+}]$ can strongly affect both the solution conformation of osteocalcin (Figure 3) and its affinity for HA (Figure 8). Normal extracellular (serum) concentrations of ionized Ca^{2+} (1.0–1.5 mM) suggest that circulating osteocalcin would be predominantly helical (Figure 3) although extrapolation to the in vivo situation may involve some complications. Physiological fluctuation or pathological perturbation of $[Ca^{2+}]$ could dramatically shift the helix–coil equilibrium and HA affinity of osteocalcin, allowing signaling or amplification of ionic changes by adsorption/desorption of the protein. It is predicted, for example, that hypercalcemia per se would tend to reduce the serum osteocalcin concentration.

The final significance of Ca^{2+}-dependent structure in osteocalcin awaits discovery of the function of this protein. Recent descriptions of high molecular weight precursors in the biosynthetic pathway of osteocalcin (Lian & Heroux, 1979; Hauschka, 1979a; Nishimoto & Price, 1980; Hauschka et al., 1982b) must be integrated into an understanding of this function. The inferred participation of proteolytic cleavage in the excision of osteocalcin from its precursor suggests an additional possible role for Ca^{2+} as an activator for this cleavage process.

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