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X-ray Crystal Structure of Bovine 3 Glu-Osteocalcin

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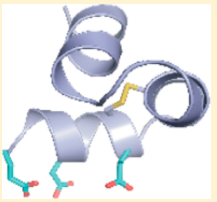
ABSTRACT: The 3 Glu form of osteocalcin (3 Glu-OCN) is increased in serum during low vitamin K intake or oral anticoagulant use (warfarin). Previous reports using circular dichroism show it is less structured than 3 Gla Ca^{2+}-osteocalcin and does not bind strongly to bone mineral. Recent studies have suggested a role for 3 Glu-OCN as a potential regulator of glucose metabolism. A G-protein-coupled receptor, GPRC6a, found in the pancreas and testes was identified as the putative osteocalcin receptor. The purpose of this study is to determine the high-resolution structure of bovine 3 Glu-OCN, using X-ray crystallography, to understand molecular interactions with mineral and the GPRC6a receptor. Diffraction quality crystals of thermally decarboxylated bovine osteocalcin were grown, and the crystal structure was determined to 1.88 Å resolution. The final refined structure contained residues 17−47 and, like 3 Gla Ca^{2+}-OCN, consisted of three α-helices surrounding a hydrophobic core, a C23−C29 disulfide bond between two of the helices, and no bound Ca^{2+}. Thus, the helical structure of 3 Glu-OCN is Ca^{2+}-independent but similar to that of 3 Gla Ca^{2+}-OCN. A reduced level of mineral binding could result from a lower number of Ca^{2+} coordinating ligands on 3 Glu-OCN. The structure suggests the GPRC6a receptor may respond to helical osteocalcin and will aid in providing molecular mechanistic insight into the role of 3 Glu-OCN in glucose homeostasis.



Osteocalcin, a small, abundant noncollagenous protein synthesized by osteoblasts, contains three γ-carboxyglutamic acid (3 Gla-OCN) residues generated post-translationally in a vitamin K-dependent process.[1−3] High-resolution structures from two-dimensional nuclear magnetic resonance (NMR) and X-ray crystallography show that the binding of Ca^{2+} to the three Gla residues induces a conformational rearrangement to a more globular species, in which these residues occupy positions complementary to the Ca^{2+} sites presented in the hydroxyapatite crystal.[4−7] Consequently, *in vivo* osteocalcin is predominantly mineral-bound, with a small amount secreted into the circulation.[8−13] Several studies have demonstrated a role for osteocalcin in remodeling bone, enhancing mineral deposition, regulating crystal morphology, and increasing bone toughness and fracture resistance.[14−20]

Recent studies have focused on the unmodified (3 Glu-OCN or 0 Gla-OCN) or partially modified (1 Glu-OCN or 2 Gla-OCN) forms of osteocalcin as potential regulators of glucose metabolism.[21,22] Animal- and cell-based studies reported that 3 Glu-OCN or 1 Glu-OCN increases pancreatic β-cell proliferation, insulin secretion, and expression of adiponectin, an insulin-sensitizing hormone produced by adipocytes.[21,22] A study in mice suggested that *in vivo*, binding of insulin to the osteoblast promotes bone resorption where acidification of the bone extracellular matrix (pH 4.5) induces decarboxylation of osteocalcin to the 1 Glu form (13-Glu in mice), resulting in an increased level of insulin secretion and sensitivity.[22] Another study showed that intermittent injections of 3 Glu-OCN improved glucose tolerance and insulin sensitivity in the mouse and may prevent the development of type 2 diabetes.[23] Recent studies suggest a connection among bone, 3 Glu-OCN, and male fertility.[24] A G-protein-coupled receptor (GPCR), GPRC6A, has been identified as the putative osteocalcin receptor.[24] A newly published report indicates that 3 Glu-OCN affects brain development and functions via a receptor other than GPRC6A, suggesting there are several receptors for the molecule.[25]

Both partially modified (1Glu-OCN and 2 Glu-OCN) and unmodified (3 Glu-OCN) forms of osteocalcin are present in humans. A recent study in genetically altered mice suggested that bone resorption by osteoclasts contributes to the partial modification of osteocalcin to the 1 Glu-OCN form and proposed that this may occur in humans, as well.[22,26] Older studies demonstrated that bone and serum osteocalcin are partially modified or unmodified as a consequence of suboptimal vitamin K intake in the population.[27] Concentrations of partially or unmodified forms of osteocalcin are increased in human serum with vitamin K depletion and decreased with vitamin K supplementation.[28,29] Vitamin K deficiency can also be produced with oral anticoagulants (warfarin and coumarin) that act as vitamin K antagonists by inhibiting the epoxide reductase responsible for vitamin K recycling. A study in rats reported a shift in osteocalcin from 3 Gla-OCN to the 3 Glu-OCN form 3 h after warfarin administration.[30] It was reported that warfarin is the most widely used anticoagulant in the world, with more than 30 million prescriptions written annually in the United States.[31] This indicates that a large percentage of the population is likely to have a significant amount of 3 Glu-OCN and partially modified osteocalcin in their serum.

Despite the reported biological importance of 3 Glu-OCN, little structural information is available. A previous circular

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dichroism study reported that in the presence of Ca^{2+}, the 3 Glu-OCN molecule contained significantly less α-helical structure than the 3 Gla form.[6] Furthermore, the 3 Glu-OCN molecule differed from 3 Gla-OCN in that it did not bind Ca^{2+},[32] was not a mineralization inhibitor,[32] and exhibited weakened binding to hydroxyapatite.[30,33] Here we report the first high-resolution structure of unmodified bovine osteocalcin (3 Glu-OCN) using X-ray crystallography and compare it to the previously reported structure of the 3 Gla Ca^{2+}-OCN. The structural information reported here may help explain previously reported observations and provides mechanistic insights into the interaction of both 3 Glu-OCN and 3 Gla Ca^{2+}-OCN with physiologically important cell surface receptors.

METHODS

Bovine osteocalcin was purified from bovine bone powder as previously described.[34] The purity of the protein was determined by polyacrylamide gel electrophoresis, reverse-phase high-performance liquid chromatography, and amino acid analysis. Dry bovine 3 Gla osteocalin was decarboxylated as previously described.[32] Briefly, 2−5 mg of dry bovine 3 Gla protein was dissolved in 0.05 M HCl and freeze-dried in a vacuum reaction tube. Decarboxylation was accomplished by heating the dried protein *in vacuo* at 110 °C for 3.0 h.[32] For crystallization studies, the lyophilized 3 Glu protein was resuspended in 20 mM NaCl and 10 mM $CaCl_2$ (pH 7.0) at a concentration of 13.3 mg/mL.

Protein Crystallization. Diffraction quality crystals were grown using the sitting drop vapor diffusion method by mixing 1 μL of protein and 1 μL of reservoir solution and equilibrating samples against the corresponding reservoir solution. The reservoir solution contained 2.5 M ammonium sulfate and 0.1 M Bis-Tris propane (pH 7.0). Crystals reached maximal size in 4 weeks.

Collection of X-ray Diffraction Data and Crystallographic Refinement. Crystals of 3 Glu-OCN with dimensions 0.2 mm × 0.2 mm × 0.2 mm were supplemented with 0.8 M lithium sulfate as a cryoprotectant and flash-cooled in liquid nitrogen. Diffraction data were collected on a Quantum 315 CCD detector (Area Detector Systems Corp.) with 1.08 Å wavelength radiation on the X29A beamline [National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, Upton, NY]. Intensities were integrated using HKL2000 and reduced to amplitudes using SCALEPACK2MTZ (see Table 1 for statistics).[35,36] The structure was determined by molecular replacement using PHASER.[37] Model building and refinement were performed with REFMAC and COOT.[36,38] The quality of the final structure was verified with composite omit maps, and the stereochemistry was checked with MOLPROBITY.[39] The LSQKAB and SSM algorithms were used for structural superimpositions.[36,40] Figures were prepared using PyMOL (Schrödinger, LLC).

RESULTS

Crystal Structure of 3 Glu-OCN. Diffraction from the 3 Glu-OCN crystals was consistent with space group $P4_1$ with two 3 Glu-OCN molecules per asymmetric unit and the following unit cell dimensions: $a = b = 42.76$ Å, $c = 37.99$ Å, and $\alpha = \beta = \gamma = 90°$. The structure of 3 Glu-OCN was determined using the molecular replacement method (PHASER[37]) with the structure of porcine osteocalcin [Protein Data Bank (PDB) entry 1Q8H] as a starting model. The two chains in the asymmetric unit are related by a local 2-fold axis, but according to PISA analysis within COOT,[38] the limited subunit interface is not predicted to support dimer formation in solution. The two chains are very similar (rmsd of 0.31 Å, among all C_α atoms), with only some side chain deviations (see Figure 1B). The final refined structure contains residues Glu 17−Gly 47 in chain A, Glu 17−Pro 48 in chain B, and 30 water molecules. Residues 1−16 and 49 are not represented in the refined electron density map, consistent with the unstructured, unconstrained N-terminal region (residues 1−15) previously reported for the structure of bovine 3 Gla Ca^{2+}-OCN determined by solution NMR.[4] The structure of 3 Glu-OCN (Figure 1) consists of three α-helices (H1−H3) with only one residue, Asn 26, outside the α-helical region of the Ramachandran plot. The asparagine side chains at the N-terminus of a helix are known to be stabilizing as a consequence of hydrogen bonds formed with exposed main chain amides. This may be the case in our structure in chain A, but in chain B, the orientation of Asn 26 is distorted by crystal contacts. The possible stabilizing interaction of the Asn 26 side chain is shown in Figure 1A. Helices 1 and 2 cross at an angle of aproximately 120° and are linked by a disulfide bond between Cys 23 and Cys 29. The hydrophobic core of the 3 Glu-OCN molecule is located among the three helices as shown in Figure 1A. The three functionally important Glu side chains extend from helix 1 (H1) and are fully solvent-exposed on a common face of the helix, consistent with direct contacts that would support mineral binding.

The structures of 3 Glu-OCN and porcine 3 Gla Ca^{2+}-OCN (previously determined by X-ray crystallography[7]) are very similar (Figure 1B,C), with an amino acid sequence identity of

Table 1. Data Collection and Refinement Statistics for 3 Glu-OCN

Data Collection	
beamline	NSLS-X29A
wavelength (Å)	1.08
resolution limits (Å)	20−1.88
no. of observed reflections	39344
no. of unique reflections	5668
completeness (%)	99.9 (98.9)[a]
R_{merge}[b]	0.063 (0.663)[a]
Refinement	
no. of protein non-hydrogen atoms	525
no. of water molecules	30
R_{cryst}[c]	0.174 (0.176)[a]
R_{free}[c]	0.245 (0.290)[a]
average B factor (Å^2)	41.1
rmsd from ideality	
bond lengths (Å)	0.010
bond angles (deg)	1.30
torsion angles (deg)	22.2
Ramachandran plot (%)	
core	100.0
allowed	0.0
generous	0.0

[a]Values in parentheses indicate statistics for the high-resolution bin. [b]$R_{merge} = \sum_j\sum_j|I_j(hkl) - \langle I(hkl)\rangle|/\sum_j\sum_j|\langle I(hkl)\rangle|$, where I_j is the intensity measurement for reflection j and $\langle I\rangle$ is the mean intensity over j reflections. [c]$R_{cryst}/R_{free} = \sum||F_o(hkl)| - |F_c(hkl)||/\sum|F_o(hkl)|$, where F_o and F_c are observed and calculated structure factors, respectively. No σ cutoff was applied. Five percent of the reflections were excluded from refinement and used to calculate R_{free}.

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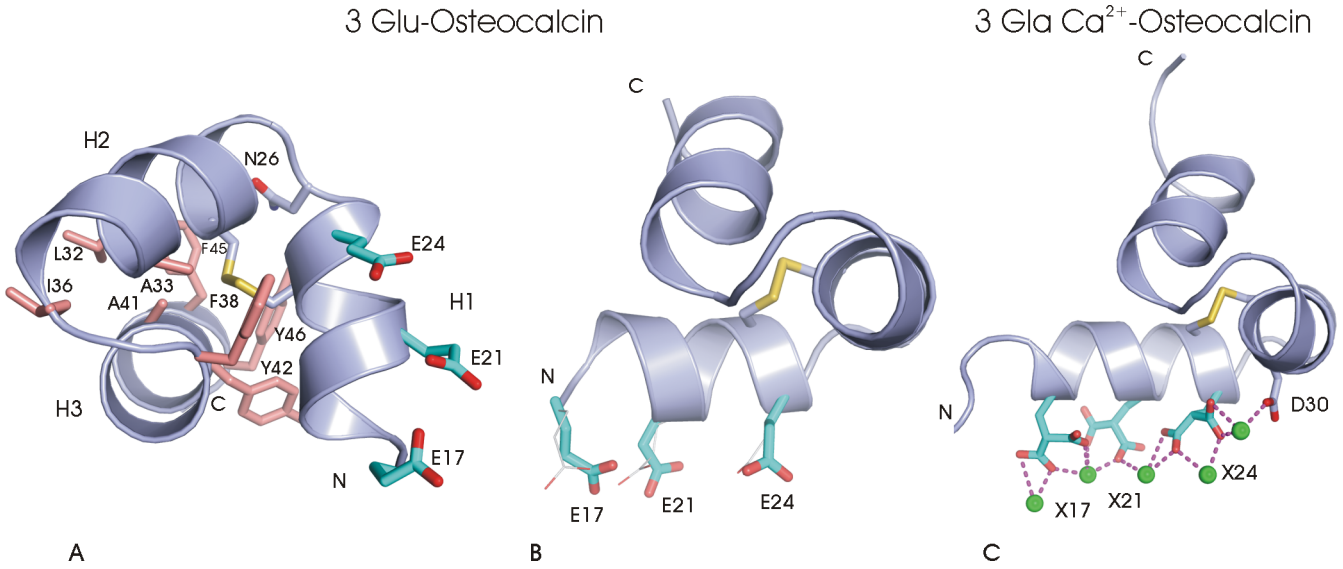

Figure 1. (A) Bovine 3 Glu-OCN structure showing the relative orientation of the main helices (H1−H3) as well as the disulfide bridge (C23−C29, yellow bond) and the hydrophobic side chain interactions within the hydrophobic core (salmon). The Asn 26 residue is shown capping the N-terminus of helix 2. The structural comparison between bovine 3 Glu-OCN (B) [where the Glu side chains are colored cyan (thin bonds, in chain B)] and porcine 3 Gla Ca^{2+}-OCN (C, PDB entry 1Q8H). The calcium ions are shown as green spheres, and coordinating ligands were determined in the previously published X-ray crystal structure.[7]

97% in the structured regions of the molecules (residues 17−47) and an rmsd of 0.61 Å (among all C_α atoms). Important differences between the two species are the fact that the 3 Glu-OCN molecule does not show bound Ca^{2+} and the fact that the 3 Glu-OCN molecule is monomeric at protein concentrations used for crystallography. In contrast, 3 Gla-OCN possesses additional COOH groups as a consequence of the post-translational modification of the side chains of E17, E21, and E24, resulting in Gla residues (X) that coordinate Ca^{2+} in solution and in the mineral phase.[7,32] NMR and crystallography studies reported that 3 Gla Ca^{2+}-OCN formed a Ca^{2+}-dependent dimer, with the dimer interface at the Ca^{2+} binding sites (K_d for dimerization of 160−200 μM).[4,7] However, the *in vivo* osteocalcin concentrations are much lower, in the nanomolar range,[11] suggesting that the monomer is the physiologically relevant oligomeric state. The number and positions of the Ca^{2+} ions in Figure 1C are those reported in the dimer interface of the porcine Ca^{2+}-OCN X-ray structure[7] and also the predicted Ca^{2+} binding sites for the monomer interacting with the bone crystal surface.[7] This previous study reported three tight and two weaker Ca^{2+} binding sites.[7] The important difference between the two structures is that apo 3 Glu-OCN adopts a highly helical structure that is independent of Ca^{2+}, whereas past studies have shown apo 3 Gla-OCN is an unstructured random coil, requiring millimolar levels of Ca^{2+} to adopt the helical structure.[5,6]

DISCUSSION

We have determined the first high-resolution structure of bovine 3 Glu-OCN using X-ray crystallography. There is considerable sequence homology among all species of osteocalcin and several conserved regions, including the Glu helix (H1), as well as the two other helical regions of the molecule (H2 and H3). Bovine osteocalcin shares 97% sequence identity with human osteocalcin, as well as a porcine ortholog, in the structured portion of the molecule (residues 17−47). Thus, the structure reported here is likely to be representative of 3 Glu-OCN in all of these species. Murine osteocalcin has a sequence less identical to that of the bovine form (61%); however, secondary structure analysis programs and homology modeling predict the same helical regions in this protein.[41] Conservation of key hydrophobic residues also suggests the presence of a hydrophobic core and a similar structure in murine 3 Glu-OCN.

Previous structural studies of 3 Glu-OCN were limited to circular dichroism (CD) spectra,[6] which predicted a rather unstructured, less helical (18−26%) molecule as compared to 3 Gla Ca^{2+}-OCN (38%).[6] These values contrast the significantly greater helical contents observed in our crystal structure of 3 Glu-OCN in this study (57%) or in the crystal structure of 3 Gla Ca^{2+}-OCN (57%) reported previously.[7] For a number of helical proteins (myoglobin, hemoglobin, lysozyme, calmodulin, etc.), the helical content predicted by CD is significantly lower than that exhibited by X-ray crystallography or Fourier transform infrared solution spectroscopy.[42] Inaccuracies in CD measurements can arise from the presence of scattering particles or protein concentrations that are too high or too low. Also, data collected in buffers at physiological ionic strength (0.150 M NaCl) can show a signal fluctuation around 190 nm due to Cl^- ions that compromise the accuracy of the protein secondary structural prediction.[42] Variability and overlap in the positions of the protein absorption bands as well as limited reference data reflect additional challenges for the empirical analysis of CD data.[42] It is likely that these factors contribute to the discrepancy in secondary structure content determined by CD spectroscopic and crystallographic methods.

The structure of 3 Glu-OCN is very similar to that of 3 Gla Ca^{2+}-OCN. The requirement for millimolar concentrations of Ca^{2+}, or other divalent metal cations, to induce the conformational change in 3 Gla-OCN is most likely related to the need to neutralize the electrostatic repulsion in this region before the molecule can properly fold into a helix. The 3 Glu molecule has a significantly lower negative charge, and glutamic acid has a strong propensity to adopt a helical conformation, supporting a

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well-folded and compact structure in the absence of Ca^{2+}. The structure of 2 Gla Ca^{2+}-OCN (Glu 13, mouse; Glu 17, bovine and human) has not yet been reported but has a sequence similar to that of 3 Gla Ca^{2+}-OCN except one fewer negative charge. The Ca^{2+} dependence of 2 Gla-OCN is unknown but may lie somewhere between the 3 Glu- and 3 Gla Ca^{2+}-OCN molecules.

Previous reports have shown that unmodified osteocalcin (3 Glu OCN) does not bind Ca^{2+},[32] is not a mineralization inhibitor,[32] and exhibits a reduced level of binding to hydroxyapatite and bone.[30,33] Because the tertiary structures of 3 Gla Ca^{2+}-OCN and 3 Glu-OCN are similar, the differences in Ca^{2+} binding properties in solution and in the mineral phase are most likely due to the reduced number of Ca^{2+}-coordinating ligands on 3 Glu-OCN. The Ca^{2+} ion requires six coordinating ligands that can be donated from the protein and H_2O molecules. 3 Gla-OCN would have more Ca^{2+} ligands contributed from carboxyl groups on the protein, rather than from H_2O molecules, leading to stronger Ca^{2+} binding in both the solution and mineral phases. This would explain why 3 Glu-OCN is not an effective inhibitor of hydroxyapatite crystallization from metastable solutions of Ca^{2+} and HPO_4^{2-}[32] and does not bind as well to hydroxyapatite.[30,33]

Recent studies have suggested that the unmodified form, 3 Glu-OCN, and partially modified form of osteocalcin, 1 Glu-OCN, are bone-derived multifunctional hormones.[21,22,24] *In vivo* studies in mice and *in vitro* studies in cells reported that the 3 Glu-OCN and 1 Glu-OCN molecules increased the level of pancreatic β-cell proliferation, insulin secretion, insulin sensitivity, and energy expenditure.[21,22] Data from cellular and genetic experiments also suggested that 3 Glu-OCN plays a role in male fertility by promoting the synthesis of testosterone by Leydig cells in the testes.[24] Further experiments identified the receptor for 3 Glu-OCN as a G-protein-coupled receptor, GPRC6A, expressed in Leydig cells in the testes.[24] A later study provided evidence that GPRC6A was the receptor for 3 Glu-OCN in mouse pancreatic β cells and in the mouse pancreas *in vivo*.[43]

GPRC6A belongs to the C family of G-protein-coupled receptors that mediate intracellular responses to diverse extracellular stimuli.[44] This receptor is present in most tissues except intestines and parathyroid gland and may regulate multiple processes.[45−47] Neither the structure of GPRC6A nor the molecular mechanism for osteocalcin binding has been reported. However, an *in vitro* study investigating the ligand specificity for GPRC6A stimulation suggests the receptor may respond to osteocalcin in a helical conformation.[47] It was shown that 3 Gla-OCN did not stimulate GPRC6A at low Ca^{2+} concentrations (<1 mM) but exhibited a dose-dependent stimulation at Ca^{2+} concentrations from 1 to 5 mM.[47] The maximal stimulation occurring at 5 mM Ca^{2+} is consistent with the Ca^{2+} concentration shown to induce the maximal helical conformational change in 3 Gla-OCN.[5,6] This is further supported by the ability of 3 Gla-OCN and millimolar concentrations of either Sr^{2+} or Mg^{2+} (divalent cations shown to induce α-helical structure in 3 Gla-OCN) to stimulate GPRC6A.[6,47] The apo form of 3 Gla-OCN is an unstructured random coil that becomes increasingly helical upon Ca^{2+} or divalent metal ion complexation.[5,6] The three Gla residues bind Ca^{2+} rather weakly with K_d values in the millimolar range, and an NMR titration showed that three Ca^{2+} ions per molecule are needed to achieve the maximal structural change.[4,48] Thus, at Ca^{2+} concentrations of 0−1 mM, a large fraction of the molecules would be only partially complexed with Ca^{2+} and significantly less helical than at 5 mM Ca^{2+} as shown by CD titrations.[5,6]

Further support for a role of helical osteocalcin in the stimulation of GPRC6A comes from the structure of 3 Glu-OCN reported in this study, which exhibits the same α-helical conformation as 3 Gla Ca^{2+}-OCN. The 3 Glu-OCN molecule was reported to stimulate GPRC6A in pancreatic and testes cells.[24,43] Several studies have shown that 3 Glu-OCN is a molecule involved in insulin signaling, whereas 3 Gla-OCN is not effective.[21,22] If GPRC6A responds to helical osteocalcin, then *in vivo* in serum or in media with Ca^{2+} concentrations of 1 mM, the receptor would be more responsive to helical 3 Glu-OCN as opposed to a less structured 3 Gla Ca^{2+}-OCN. It is possible that 3 Glu-OCN may be involved in cell receptor interactions where helical osteocalcin is required and the Ca^{2+} concentration is low, while 3 Gla-OCN affects bone and mineral crystal properties through its ability to bind divalent metal cations in both mineral and solution. The structure reported in this study may aid in the development of a molecular mechanism for the interaction of 3 Glu-OCN and 3 Gla Ca^{2+}-OCN with cell surface receptors and may shed light on the role of unmodified 3 Glu-OCN in glucose homeostasis.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Gla, γ-carboxyglutamic acid; Glu, glutamic acid; rmsd, root-mean-square deviation.

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