Structure, Activity, and Distribution of Fish Osteocalcin*

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Osteocalcin (bone Gla protein) is an extracellular matrix protein synthesized by osteoblasts that is a marker of bone. Osteocalcin probably originated in the ancestors of Teleostei or bony fish and of the Tetrapoda or amphibians, reptiles, birds, and mammals. We have characterized the Cyprinus carpio (carp) osteocalcin for mineral binding to hydroxyapatite, amino acid sequence, and extent of secondary structure. Hydroxyapatite binding is enhanced in the presence of calcium. The α-helical content of teleost osteocalcin increases and β-sheet structure decreases upon calcium binding, similar to findings in calf osteocalcin. The gene structure and primary sequence of prepro-osteocalcin from 2 pufferfish compared with carp shows that there are many conserved features in teleost osteocalcin genes. Using an immunoassay for carp osteocalcin, we determined that the relative content of osteocalcin is highest in dorsal fin spines and other bones and lowest in scales. The carp osteocalcin antibodies, cross-reactive to other species of fish, were used to study the role of osteocalcin in teleost model systems.

Bone contains a vitamin K-dependent protein containing γ-carboxyglutamic acid (Gla)1 called osteocalcin (Oc) or bone Gla protein (1, 2). Bony fishes (Teleostei) and land vertebrates (Tetrapoda) contain the protein Oc (2). Protein sequence comparisons reveal the highest sequence conservation in the Gla-containing domain (3, 4). Gla and the conformation conferred by the Gla domain appear necessary for Oc to bind to hydroxyapatite (HA) (5, 6). Calcium binding causes a conformational change that coincides with increased affinity for hydroxyapatite (7, 8).

Bone mineral binding, tissue distribution, and structural studies of osteocalcin have been performed almost exclusively on proteins from mammals and birds (Tetrapoda). Bony fish (Teleostei) are the most successful organisms in aquatic environments. The common ancestor of tetrapods and teleosts evolved bone over 200 million years ago and a comparison of Oc in the two orders can provide insights into the evolution of Oc structure and function. For example, the amino acid sequences of most tetrapods contain a conserved C-terminal RRFYPV sequence that is missing in teleosts, while a truncated N terminus and extended C terminus are missing in tetrapods (3, 4).

The present studies of Cyprinus carpio osteocalcin and sequences derived from other fish genome sequences (Fugu rubripes and Tetraodon nigroviridis) confirm the primary structure differences observed in osteocalcins of tetrapod and teleost. A higher proportion of carp Oc is α-helical when compared with the calcium-free bovine Oc. The studies also characterize mineral binding behavior and tissue distribution of teleost Oc using a C. carpio Oc radioimmunoassay.

EXPERIMENTAL PROCEDURES

Purification of Osteocalcin—Carp (C. carpio) bones were removed and cleaned of adherent tissue after placing in boiling water for 1–1.5 min as described previously (3). Bones were broken into 8–125 mm3 pieces, water washed for 20 min, then lyophilized. The dried bone was ground in a blender. Particles passing through a 0.425-mm sieve were washed in distilled water for 2 h; acetone-, trichloroethylene-, and acetonewashed for 1 h; and air-dried. Bone proteins were extracted with 20% formic acid (6 ml/g of bone) at 4 °C for 4 h, the supernatant filtered through a 0.8-μm Millipore Millex FF filter, then desalted by gel filtration on Sephadex G-25 equilibrated with 10% formic acid. The collected protein fraction was diluted to 5% formic acid and then lyophilized. The lyophilized proteins were dissolved in 5 μl guanidine, 0.1 M Tris, pH 8, and separated by gel filtration on Sephacryl S-200 equilibrated with the same buffer. Bones of different species were analyzed by gel filtration to estimate osteocalcin content. Final purification for sequence analysis was by high pressure liquid chromatography on a Vydac C18 reversed phase column with gradient elution on a Beckman 332 gradient liquid chromatograph equipped with a Beckman 420 Controller, Amersham Biosciences UV-1 spectrophotometer, and a single channel chart recorder. The elution gradient was initially 0.1% trifluoroacetic acid, 21% acetonitrile with a linear gradient to 0.1% trifluoroacetic acid, 56% acetonitrile over 25 min with a flow rate of 1.5 ml/min. Protein peaks were detected by absorbance at 280 nm. The single peak eluting at ~20 min was manually collected. Purity of osteocalcin was established with recovery of a single Coomassie Blue-stained band on a 15% polyacrylamide native gel, which coincided with the major Gla-containing protein found by dithobenezensulfonic acid (DBS) staining for Gla-containing proteins, as described (3, 9).

Protein Sequence—Purified Oc was digested by cyanogen bromide (CNBr), BNPS skatole, or Staphylococcus aureus V8 protease; the peptides were separated by reversed phase HPLC using a modified gradient of 7–56% acetonitrile over 35 min. Samples were lyophilized in microcentrifuge tubes for sequence, amino acid composition, and mass spectral analyses. Intact protein and proteolytic peptides were sequenced in a Porton Instruments sequenator as described previously (3). Amino acid analysis of Oc and peptides was performed on a Beckman system 6300, and carboxypeptidase Y digestion was performed by established methods (3). The C-terminal amino acid sequence was deduced by tandem mass spectroscopy with CID (collisional inactivation decomposition) with CO2 as the bombarding gas using the purified C-terminal S. aureus V8 protease fragment of osteocalcin (10, 11).

Conformational Studies by Circular Dichroism—Purified Oc was made calcium-free (apoOc) by incubating Oc (1 mg/ml) in 5 mM EDTA, pH 7.4, for 15 min at room temperature, then resalting over a...
50-mL Sephadex G-25 F column equilibrated with 5 mM ammonium bicarbonate to separate apoOc from EDTA and Iodoacetamide. The collected apoOc was lyophilized and resuspended in 1× Tris, pH 7.33, and diluted to 4 and 5 mM. Bovine and carp Oc protein concentrations were estimated as 1 unit of 230 nm absorbance as equivalent to 200 μg/mL. Circular dichroism spectra were collected from 195 to 300 nm with data collection for 10 s averaging in 1-nm increments with 2 repeats at 25 °C. CaCl₂ (1 mM) was added to adjust calcium concentration to 5 mM, and the CD spectrum collected again. An Aviv model 62S spectropolarimeter equipped with electronic temperature control and a 1-mm pathlength quartz cuvette was used for circular dichroism studies. Mean molar ellipticity was calculated by \([\theta] = (1000/\lambda)\text{cm}^2\text{dmol}^{-1}\); units are deg cm²/decimol (° is ellipticity in millidegrees, c is concentration mol/liter, n is number of amino acid residues per protein, and I is pathlength in cm). The estimated secondary structure was determined from the molar ellipticity from 200 to 240 nm for each unknown and the K2d program for estimation of secondary structure from circular dichroism data, which gives estimated structure and the error estimate (kal.cal.egr.es/k2d/k2d.html) (12, 13).

Radioimmunoassay for Carp Osteocalcin—Two New Zealand White rabbits were inoculated with a mixture of Freund's Adjuvant and purified osteocalcin (500 μg) at monthly intervals. All experiments used a 1:16,667 final dilution of M47 4/12 antiserum. Standard curves were made from serial dilution of purified carp Oc using 5 units of absorbance at 230 nm to be 1 mg/mL. Iodination of carp Oc, assay diluent, buffers, and conditions were identical to those published (14–16). All samples were assayed in triplicate. After determining bound radioiodinated Oc tracer (Packard Auto-Gamma Model 5002, Packard Instrument Co.) the concentration of unknown was determined from a spline-fit curve using Packard Cobra software (16).

Extraction and Assay of Proteins from Mineralized Tissues—The Oc content was determined from the supernatant of 10% formic acid extracts neutralized and diluted as described (16). Cross-reactivity of the assay was determined from serial dilutions of 10% formic acid extracts of carp, salmon, trout, and carp. Serum albumin (Grand Island Biological fraction V) and was saturated with hydroxyapatite. All assays were performed in 575 μl with different amounts of hydroxyapatite, osteocalcin, and added cations or antibodies. Incubations were done for 20 h at 4 °C in a 1.5-mm screw cap microcentrifuge tubes with constant gentle rotation. Unbound Oc was assayed from the supernatant after spinning for 3 min at top speed in a Beckman Microfuge 11. 5 μl of anti-osteocalcin antisera (1:115 dilution) or an equal amount of normal rabbit serum were added to test the effect on Oc-hydroxyapatite binding. 125I-Osteocalcin was diluted to 10 ng/ml with unlabeled Oc and the bound and free fractions determined by radioactive counts. Binding was assessed by nonlinear regression analysis using Graphpad Prism 3.0 software (17).

Determination of F. rubripes and T. nigroviridis Oc Sequences and Flushing DNA Sequences from Genomic Sequences—The Fugu (tiger pufferfish) exon sequences were identified by TBLASTN search using sequences corresponding to carp protein to search the Fugu data base at the Joint Genome Institute (JGI) at baltimore.igi-psf.org, finding matching sequences within the 8156 bases of scaffold 10600 (version 1). The Sparus aurata Oc gene sequence was used to probe for homologous regions of prope-Oc of exons 1 and 2. Determination of the intron boundaries were supported by the presence of consensus splice sites. The Tetraodon (spotted pufferfish) exon sequences were identified by BlastN 2.2.31 with the T. nigroviridis WGS Trace data base to be in 2 sequences, gnl/ti/100718496 G14P616840RE3.E2.T0 (exon 1 and upstream elements) and gnl/ti/100777902 G14P612432RE6.E2.T0 at www.ncbi.nlm.nih.gov/blast/Blast.cgi. Consensus sequence searches and confirming translations of exon sequences were performed with the program Gene Jockey 2. SignalP V1.1 identified signal peptides and likely cleavage sites (at www.cbs.dtu.dk/services/SignalP/) (18).
Carp ApoOc and Calcium-bound Oc Has Greater Percentage of α-Helix—The circular dichroism spectrum of carp apoOc indicates that carp apoOc has a higher percentage of α-helical secondary structure compared with either bovine apoOc or calcium-bound bovine Oc (Fig. 2, Table I). The bovine Oc spectrum undergoes a change in the molar ellipticity of bovine osteocalcin at 222 nm as reported previously (8). Spectral analysis showed a change from 8 to 9% α-helix content. Carp Oc exhibited a higher percentage of α-helix than bovine Oc in both the calcium-bound form with 5 mM calcium and the apoOc form without added calcium. ApoOc was prepared by gel filtration desalting from solutions containing 5 mM EDTA as described in “Experimental Procedures.”

Antibody for C. carpio Oc and Radioimmunoassay—Both rabbits injected with carp Oc produced antibody after the second challenge. Antiserum M47 4/12 was chosen for use in the radioimmunoassay after analysis of several sera for antibody titer. The RIA had a detection limit of 0.1 ng. Analysis of partially purified Oc from other fish species showed that the Oc from I. bubalus (buffalo), and C. idella (grass carp) cross-reacted completely, as they exhibited a dose dependence similar to that of carp extracts, and competed completely with antibody binding to 125I-carp Oc when added at >300 ng. T. aurea was partially recognized, but bluegill was poorly cross-reactive (Fig. 3A). The antisera recognized both the 1–29 N-terminal and 31–48 C-terminal CNBr peptides, indicating that there are at least 2 epitopes, one in the N-terminal half of the Oc region and one in the 31–48 C-terminal half. Both peptides inhibit binding to 1–48 Oc but neither proteolytic peptide alone inhibited binding completely (Fig. 3B).

Distribution of Oc in Mineralized Tissues—The amount of Oc in different mineralized tissues was determined by radioimmunoassay and expressed per dry weight of tissue or per unit of mineral, estimated by phosphorus assay (Table II). The level of Oc was highest in skeletal bones such as the dorsal spines, vertebral projections, and ribs. Oc levels were lower in the operculum than in any skeletal bone. Fish dermal scales were especially low in Oc, containing 30–50 times less than other mineralized tissues tested, confirming previous findings in bluegill (3).

Osteocalcin Hydroxyapatite Binding Is Enhanced by Calcium—The HA binding of carp osteocalcin is enhanced in the presence of calcium ions. Fig. 4 shows Oc-HA binding in the presence and absence of 2 mM calcium. The equilibrium dissociation constant $K_d$ of carp Oc was 22 μM while the $K_d$ with calcium was 11 μM. The $K_d$ values were calculated by nonlinear
TABLE II

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Oc/bone</th>
<th>Oc/P</th>
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</thead>
<tbody>
<tr>
<td>Dorsal spines</td>
<td>2.385</td>
<td>10.15</td>
</tr>
<tr>
<td>Vertebral projections</td>
<td>2.197</td>
<td>7.71</td>
</tr>
<tr>
<td>Rib bone</td>
<td>1.835</td>
<td>7.49</td>
</tr>
<tr>
<td>Operculum</td>
<td>1.504</td>
<td>5.81</td>
</tr>
<tr>
<td>Scales</td>
<td>0.0528</td>
<td>0.302</td>
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</tbody>
</table>

FIG. 4. Hydroxyapatite binding by carp osteocalcin. The binding isotherm of purified carp Oc for hydroxyapatite in the absence of added calcium (open squares); binding in the presence of 5 mM calcium (closed circles). Error bars represent S.D. Experiments were performed as described under "Experimental Procedures." The inset shows the Scatchard analysis. Results of the average of duplicate experiments are shown.

FIG. 5. S. rubripes osteocalcin gene. Fugu gene sequence and the available 5′-flanking region. The figure shows the reversed and complemented sequence of scaffold 10600 consisting of 8156 nucleotides. Nucleotide 1 corresponds to 8156 in scaffold for the sequence shown. The assignment of introns was based on the presence of intron consensus sequences at the 5′- and 3′-end of introns.

A TBLASTN search of the genome data base revealed that scaffold 10600 contains the Fugu osteocalcin gene (Fig. 5). The mature Oc protein is encoded by 2 exons, exons 3 and 4, with exon 4 containing a stop codon after the C-terminal phenylalanine. Nucleotide 2075–2080 mark the consensus polyadenylation sequence. Putative exon 1 contains the signal sequence and the beginning of the intracellular pro-Oc. A short putative exon 2 contains pro-Oc sequence. The assignment of exons is supported by the presence of the TATA box, CCAAT box, and OSE1 elements upstream of exon 1, and by homology to the S. aurata, mouse, and chicken Oc genes. The exon assignment is also supported by the presence of the consensus splice site sequences at the 5′- and 3′-end of introns.

Alignment of the known teleost prepro sequences indicates significant homology between the S. aurata gene sequences, plus the presence of consensus splice site sequences at the 5′- and 3′-end of introns.
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*Tetraodon nigroviridis* Osteocalcin gene. A survey of the available regulatory sequences from the *T. nigroviridis* osteocalcin gene. Four exons and three introns are present in the gene as found in all other species. The initiator methionine is indicated with a solid line, and the predicted amino acid sequence is shown above the coding sequence. Intron boundary consensus sequences are marked in bold. Asterisks indicate identities, dashes indicate conservative replacements, and dashes indicate gaps for best alignment. The cleavage between pro and mature protein is indicated as a line. The cleavage between pro and mature protein is indicated as a line. The cleavage between pro and mature protein is indicated as a line.

**Fig. 6.** *T. nigroviridis* osteocalcin gene. *Tetraodon* gene sequences contained in 2 short DNA fragments. The figure shows the reversed and complemented sequence of the 716 nucleotide-long fragment gnt/ti/100718496 G41P615684/0RE3.T0 (exon 1 and upstream elements), with nucleotide 1 corresponding to 716. Below the dashed line, the 740 nucleotide-long sequence of gnt/ti/100777902 G41P6154926RE5.T0 (exons 2, 3, and 4) is shown. Nucleotides are numbered in the left margin, and the predicted amino acid sequence is shown above the coding sequence. Intron boundary consensus sequences are marked in bold. Asterisks indicate identities, dashes indicate conservative replacements, and dashes indicate gaps for best alignment. The cleavage between pro and mature protein is indicated as a line prior to the AAG in the N terminus of the mature, secreted Oc of *T. nigroviridis*. **FIG. 7.** Comparison of sequences from teleosts and tetrapods. A, alignment of the mature Oc from six teleost and four tetrapods. Carp, *Fugu*, and *Tetraodon* are new sequences derived from the current study. Sequences are aligned N-terminal amino acid of the mature, secreted form of human Oc chosen as the starting point with spaces every 10 residues. CAR, carp (*C. carpio*); FUG, fugu (*F. rubripes*); TET, fresh-water puffer (*T. nigroviridis*); BLG, bluegill (*L. macrochirus*). Note that Gla residues are not distinguished from Glu in this figure. B, alignment of prepro sequences of Oc from the *Sparus* (20) with *Fugu* and *Tetraodon*. The initiator methionine is indicated with a number 1 below the sequences. Asterisks indicate identities, lines indicate conservative replacements, and dashes indicate gaps for best alignment. The cleavage between pro and mature protein is indicated as a space prior to the AAG in the N terminus of the mature, secreted Oc of the three fish. Analysis of sequences by Salign predicts the N terminus is a signal peptide with cleavage between Ser20 and Met20. **DISCUSSION**

Carp osteocalcin is an abundant component of carp rib bone comprising over 35% of the total extractable proteins. Carp osteocalcin is a polypeptide of 45 amino acids, highly homologous to the other teleost osteocalcins. It also has highly conserved regions in common with tetrapod osteocalcin, including the gla domain with an invariant pair of cysteine residues, which form a disulfide bond in the mature Oc (19). A region near the C terminus of the mature, secreted Oc from *T. nigroviridis* is a signal peptide with cleavage between Ser20 and Met20. Searches of the available *Danio rerio* data base have been negative as yet, except for portions of exon 4.

Upstream elements found include TATA and CCAAT boxes. *Fugu* contains an upstream consensus OSE1 element that binds an osteoblast-specific transcription factor present in osteoblasts called OSF (20). *Tetraodon* regulatory sequences upstream of exon 1 could not be identified, as an overlapping DNA fragment was not available at this time. Similarly, the inability to identify an overlapping fragment containing downstream sequences after exon 4 precluded...
identification of a polyadenylation site for *Tetraodon*, although one was noted in *Fugu*.

Antibodies raised are cross-reactive to a number of species, which should facilitate future studies of osteocalcin metabolism in teleosts. The assay cross-reacts completely with the osteocalcin from *I. bubalus* (smallmouth buffalo, Fig. 4) with which it shares very high homology with only 3 amino acid differences. Furthermore, the assay is completely cross-reactive with *C. auratus* (goldfish), a commonly used teleost model. Immunoreactive osteocalcin content varies in different mineralized tissues of carp with dorsal spines highest and scales the lowest. This result confirms assessment of osteocalcin content observed in bluegill with non-immunochemical methods (3).

Carp apoOc appears to have a higher content of α-helix in comparison to tetrapod apoOc, 24% compared with 8%. Conversely, bovine apoOc has 46% β-sheet compared with 19% in carp. The addition of calcium has been reported to induce conformational changes in osteocalcin, which is correlated with a calcium-dependent increase in hydroxyapatite affinity (8). The change in structure of osteocalcin may similarly account for the increased hydroxyapatite affinity of carp Oc compared with apoOc, although the change in conformation for carp is very small, as measured by circular dichroism. The high content of α-helical structure may make carp Oc a good candidate for two-dimensional NMR studies to determine the Oc solution structure.

Oc is the most abundant noncollagenous bone protein of most teleosts. Oc is the dominant protein component of the acid extracts of carp, bluegill, walleye, grass carp, tilapia, and buffalo (fish) bone. Similar findings are reported in *Sparus* (4). One exception is channel catfish bone, which has the same level of Oc as tetrapods. A possible explanation for low levels of osteocalcin in bone may relate to the need for catfish to utilize bone as the primary source of calcium for homeostasis. Other fish primarily use scale calcium for homeostasis, but the lack of scales in catfish forces the use of bone calcium resulting in a different distribution of osteocalcin in scaleless fishes. The hypothesis that high levels of Oc is due to acellularity of fish bone has been disproven (3). The selection of fishes tested included acellular and cellular bone, but there was no correlation for Oc levels with cellularity. These results eliminate presence of embedded osteocytes in bone as a reason for high levels of Oc.

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