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MATRIX GLA PROTEIN, A NEW γ-CARBOXYGLUTAMIC ACID-CONTAINING PROTEIN WHICH IS ASSOCIATED WITH THE ORGANIC MATRIX OF BONE

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A new protein has been isolated from $CaCl_2$/urea extracts of demineralized bovine bone matrix. This protein has five to six residues of the vitamin K-dependent amino acid, γ-carboxyglutamic acid (Gla), and we have accordingly designated it matrix Gla protein. Matrix Gla protein is a 15,000 dalton protein whose amino acid composition includes a single disulfide bond. The absence of 4-hydroxyproline in matrix Gla protein demonstrates that it is not a precursor to bone Gla protein, 5,800 dalton protein which has a residue of 4-hydroxyproline at position 9 in its sequence. Matrix Gla protein also does not cross-react with antibodies raised against bone Gla protein.

Alkaline hydrolysates of bone contain high levels of γ-carboxyglutamic acid (Gla) (1,2), the vitamin K-dependent amino acid which binds Ca^{2+}. About 80% of this γ-carboxyglutamic acid is found in bone Gla protein (BGP), a 49-residue protein which is readily extracted from bone during demineralization. The remaining 20% of the Gla remains associated with the organic matrix of bone after demineralization (3,4).

While no Gla-containing proteins had previously been isolated from the demineralized organic matrix of bone, earlier studies demonstrated that matrix associated, Gla-containing proteins precede the appearance of BGP in calcifying bone (3). Much of the matrix-associated, Gla-containing protein can be dissociated from demineralized bone by treatment with denaturants such as urea or guanidine HCl. Gel filtration of such extracts from chicken bone revealed the presence of several constituents which cross-reacted with the immunoassay

Abbreviations: Gla, γ-carboxyglutamic acid; MGP, matrix Gla protein; BGP, bone Gla protein, osteocalcin.

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developed for chicken BGP (5). These unpurified constituents, which range in molecular weight from 10,000 to 85,000 daltons, were tentatively identified as precursors on the biosynthetic pathway to BGP (5).

We here report the characterization of the first matrix-associated Gla-containing protein to be purified from bone. This protein, which we designate matrix Gla protein (MGP), is a new Gla-containing protein and is not a precursor to BGP.

METHODS

MGP was purified from $CaCl_2$ urea extracts of demineralized, gelatinized bovine cortical bone in the course of investigations whose primary objective was the isolation of the bone morphogenetic protein (6). The preparations of MGP used in the present investigations were purified from the Step VIII bone morphogenetic protein fraction by hollow fiber ultrafiltration using a 10K pore size filter. Under dissociative conditions, this method permits filtration of the 14 to 15K molecular weight MGP (6). The procedures employed in the analytical sodium dodecyl sulfate polyacrylamide slab gel electrophoresis of MGP and the standards used to establish molecular weight have been described (6).

The composition of MGP was determined by amino acid analysis of samples of purified protein hydrolyzed *in vacuo* in 6N HCl at 110°C for 24 h. The Gla and Glu levels in MGP were determined by amino acid analysis of alkaline hydrolysates as described (2). To verify the identity of the putative Gla, effluent fractions corresponding to the Gla position were removed prior to ninhydrin reaction, heated at 100°C for 4 h, and then analyzed for Gla and Glu to establish that the expected degree of decarboxylation (7) had occurred. Cis and trans 4-hydroxyproline (Hyp) were measured by amino acid analysis of the acid hydrolysate of MGP using a pH 2.9 citrate buffer and a Beckman W-2 ion exchange resin (8). Control analysis of Hyp levels in BGP demonstrated that this system resolved Hyp and yielded the expected one Hyp per molecule of BGP. The number of disulfide bonds in MGP was determined by alkylation with iodoacetate before and after reduction with mercaptoethanol as described (9).

RESULTS

Matrix Gla protein was purified from urea extracts of demineralized bovine bone by procedures described recently (6). The purified protein gave a single major band upon sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (Figure 1) with an estimated molecular weight of 15,000 daltons. A gel scan showed that this band accounts for 95% of the Coumassie Brilliant Blue stain in the gel. Based on the recovery of MGP from demineralized bone, we estimate the MGP content of bone to be 0.2 to 0.5 mg MGP per g of dry bovine bone. For comparison, bovine bone contains about 2 mg of BGP per g dry weight (2).

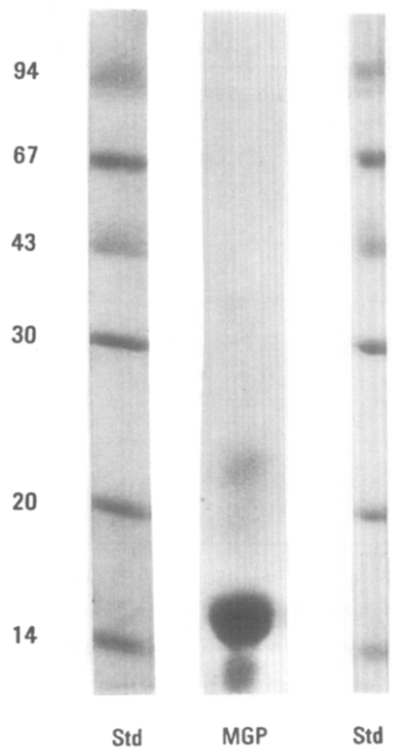

Figure 1. Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis of MGP and of molecular weight standards. Purified MGP was placed in lane 5 and molecular weight standards were placed in lanes 1 (left) and 8 (right). Electrophoresis and staining were then carried out as described (6). The stained gel was photographed and lanes 1, 5, and 8 were printed for mounting in this figure.

The amino acid composition of MGP is presented in Table I. The Gla content of MGP was confirmed by the demonstration that effluent fractions from the amino acid analyzer containing the putative Gla component were converted to the expected amount of Glu upon heating for 4 h at 100°C (7). Since carboxymethylcysteine was formed only when MGP was reduced with mercaptoethanol prior to alkylation with iodoacetate, the two cysteines of MGP must be linked in a disulfide bond. The minimal molecular weight based on one disulfide bond per molecule of MGP is 15,023, in good agreement with the 15,000 dalton molecular weight estimated from SDS gel electrophoresis (Figure 1).

Several observations demonstrate that MGP is not a precursor to BGP. The most definitive of these is the absence of 4-hydroxyproline in MGP (Table I),

TABLE I

AMINO ACID COMPOSITION OF MATRIX GLA PROTEIN

Amino Acid	Mole %	Residues/Molecule[a]
Asp	12.0	15.0
Thr	1.3	1.6
Ser	6.0	7.5
Glu[c]	10.7	13.4
Pro	4.3	5.4
Gly	3.7	4.6
Ala	9.7	12.1
Cys[b]	1.6	2.0
Val	3.7	4.6
Met	1.9	2.4
Ile	4.9	6.1
Leu	5.8	7.3
Tyr	6.4	8.0
Phe	5.1	6.4
His	2.1	2.6
Lys	3.6	4.5
Arg	12.7	15.9
Gla[c]	4.5	5.6
Hyp	<0.05	<0.05

[a] Calculated from mole % assuming 1 disulfide bond/molecule.

[b] Calculated as carboxymethylcysteine after reduction and alkylation.

[c] Calculated from analysis of alkaline hydrolysate.

an amino acid found at position 9 in the calf BGP sequence (10). Analysis of the tryptic peptides from MGP by HPLC failed to reveal peptides in the elution positions expected for any of the three tryptic peptides from calf BGP (data not shown). Sequence studies further showed that the N-terminal of MGP is blocked while the N-terminal of calf BGP is known to be free (10). Finally, the radioimmunoassay developed for calf BGP (11) detected apparent cross-reactivity only at the highest MGP level tested (Figure 2). Since a 40-fold higher level of the specific antiserum to calf BGP failed to bind any ^{125}I-labeled MGP, we conclude that this apparent cross-reactivity must reflect the presence of minor BGP contamination in the MGP preparation tested rather than true antigenic cross-reactivity between related antigenic determinants. The estimated level of BGP contamination is 0.3% in this MGP preparation.

DISCUSSION

Matrix Gla protein is the second Gla containing protein to be isolated from bone. In contrast to BGP, which is extracted from bone during demineralization, MGP is strongly associated with the collagenous bone matrix which

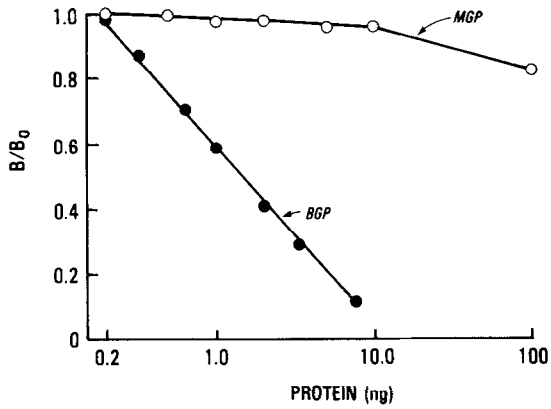

Figure 2. Radioimmunoassay of bovine BGP and its cross reactivity with bovine MGP. Relative fraction of ^{125}I-labeled BGP bound to antibody (B/B_o) at increasing levels of BGP (●) or of MGP (o). Assay procedures have been described (11).

remains after demineralization. MGP can only be extracted from this matrix with denaturants such as guanidine HCl or urea. Although the present investigation represents the first isolation of a matrix-associated, Gla-containing protein from bone, earlier studies strongly suggested the presence of such a protein. The most important of these was the demonstration that 20% of the γcarboxyglutamate content of bone is associated with the organic matrix which remains after demineralization. This matrix-associated γ carboxyglutamic acid-containing component appears in developing bone well before bone Gla protein, and was identified as a different Gla-containing bone component (3). Based on its abundance in bone, we estimate that the matrix Gla protein described here accounts for at least 50% of the γ-carboxyglutamic acid previously shown to be associated with the collagenous bone matrix. Studies are currently in progress to determine whether or not the developmental appearance of MGP precedes that of BGP, the result predicted from the developmental analysis of γ-carboxyglutamic acid in alkaline hydrolysates of demineralized bone matrix.

While the physiological function of MGP is unknown, it may be significant that MGP associates strongly with the bone morphogenetic protein (6). Denaturants are needed to extract MGP from bone matrix and to solubilize purified MGP. In contrast, purified bone morphogenetic protein is water soluble in the absence of denaturants (6). One role of MGP could therefore be to serve as an

insoluble matrix which can bind bone morphogenetic protein for delivery to the cellular targets responsible for the bone morphogenetic response.

Other possible physiological functions for MGP are suggested by the abnormalities in bone metabolism recently identified in rats maintained on Warfarin (4), a vitamin K-antagonist which blocks the formation of γ-carboxyglutamic acid. The first disorder observed in Warfarin-treated rats was the excessive mineralization of growth plate cartilage with eventual growth plate fusion and cessation of longitudinal growth (12). This disorder, which closely resembles the fetal Warfarin syndrome in humans, may be caused by an inability of Warfarin-treated animals to inhibit spontaneous propagation of hydroxyapatite crystals (12). The second disorder identified in Warfarin treated rats was an accentuation of bone mineral loss in the metaphysis of rats which were treated concurrently with $1,25(OH)_2D_3$, the active metabolite of vitamin D (13). Although the mechanism of this effect is presently unclear, the fact that the effect is only seen in rats treated concurrently with $1,25(OH)_2D_3$ suggests strongly that a vitamin K-dependent protein mediates an action of $1,25(OH)_2D_3$ on bone. With the present discovery of a second vitamin K-dependent bone protein, it is clear that abnormalities in the synthesis of both MGP and BGP must be considered as possible causes of the Warfarin bone disorders.

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