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Characterization of a γ-carboxyglutamic acid-containing protein from bone

(prothrombin/vitamin K/calcified tissues/hydroxyapatite/crystallization inhibitor)

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ABSTRACT **A γ-carboxyglutamic acid-containing protein has been purified from the calcified tissues of several vertebrates. The presence of three γ-carboxyglutamic acid residues in the bovine protein was established by alkaline hydrolysis and amino acid analysis, a method based upon studies with synthetic γ-carboxyglutamic acid. The identity of γ-carboxyglutamic acid in the bovine protein was established by mass spectroscopy on the unknown amino acid isolated from alkaline hydrolysates. The protein is extracted from finely ground bone during demineralization with EDTA, and purified from EDTA extracts by gel filtration over Sephadex G-100 and chromatography on DEAE-Sephadex. The protein has a 6800 molecular weight and an isoelectric point of pH 3.7. The amino-terminal 15 residues have been determined, and establish that this protein is not a fragment of the γ-carboxyglutamic acid-containing blood clotting factors. Similar γ-carboxyglutamic acid-containing proteins also have been purified from bovine dentine, swordfish vertebrae, and human tibia. No γ-carboxyglutamic acid can be detected in the calcified cartilage of elasmobranches, in calf epiphyseal growth cartilage, or in tooth enamel. The bovine protein binds strongly to hydroxyapatite but not to amorphous calcium phosphate, and it is a potent inhibitor of hydroxyapatite crystallization from supersaturated solutions of calcium and phosphate.**

In an impressive series of experiments (1–3) a new amino acid has recently been identified in bovine prothrombin. This amino acid is γ-carboxyglutamic acid (Gla), and it is synthesized from glutamic acid residues in a post-translational enzymatic reaction with an absolute requirement for vitamin K and bicarbonate (4). Several functions of Gla residues in prothrombin have been identified by comparing prothrombin with the abnormal protein formed when the synthesis of Gla is inhibited by vitamin K antagonists such as dicumarol. The Gla residues in prothrombin enable factor Xa to convert prothrombin to thrombin at an accelerated rate in the presence of Ca^{2+} and phospholipid (5). Gla residues are also necessary for prothrombin to bind to phospholipid vesicles in the presence of Ca^{2+}, and to bind to insoluble salts such as barium sulfate and calcium phosphate (6). From these functions of Gla in prothrombin, it is logical to search for Gla in other proteins where Gla might similarly facilitate association with membrane or crystal surfaces.

Calcified tissues represent a system in which a Gla-containing protein might be found, and Gla has indeed been reported recently in protein fractions isolated from EDTA extracts of chicken bone (7). In the present study we describe the independent discovery of a similar Gla-containing protein in extracts obtained by EDTA or acid decalcification of bovine cortical bone. The method used to identify Gla in both studies is based upon the stability of Gla under conditions of alkaline protein hydrolysis, as inferred from the alkaline stability of its structural analogues, malonate (8) and aminomalonate (7). In the work on chicken bone a presumptive Gla fraction was isolated by ion exchange chromatography from alkaline hydrolysates of the chicken bone and identified as Gla on the basis of both its conversion to glutamic acid upon heating in acid and its coelution with a similar putative Gla fraction in alkaline hydrolysates of prothrombin (7). In the present study we have used Gla synthesized by the procedure of Morris *et al.* (9) to show further that the mass spectra of the presumptive Gla from bovine bone and synthetic Gla are identical. Studies with synthetic Gla have also enabled us to show that Gla is indeed completely stable under the conditions of alkaline protein hydrolysis, and to establish its ninhydrin color factor for quantitative estimation of Gla content in proteins.

In the present work we report the chemical composition of the bovine Gla protein and the sequence of its first 15 residues, which establish that the bovine Gla protein is not a fragment of the bovine blood clotting factors. We also present evidence of a similar Gla-containing protein in most other bovine calcified tissues and in a variety of other vertebrates. The low molecular weight and high electrophoretic mobility of the main Gla protein in chicken bone (7) indicate that it is probably another member of this class of proteins. We find that the bovine Gla protein binds strongly to the mineral phase of bone, with an average stoichiometry of one Gla protein per crystal, and that the bovine Gla protein is a potent inhibitor of hydroxyapatite crystallization.

MATERIALS AND METHODS

Cortical bone was obtained from the central section of the femur of freshly slaughtered calves or cows. Bone samples were freed of marrow and connective tissue, ground to a particle size that passed through a 210-μm sieve, and washed with several changes of water for 24 hr at 4°. The bone was then dialyzed against several changes of 0.5 M EDTA, pH 8, at 4° for 8–10 days. The soluble fraction inside the dialysis sack was collected by centrifugation, dialyzed exhaustively against 5 mM NH_4HCO_3, and lyophilized. After gel filtration on Sephadex G-100 (Fig. 1), peak fractions containing the Gla protein were lyophilized and then chromatographed on a 2 × 50 cm column of DEAE-Sephadex A25 at 25° with a linear gradient in 0.1 M Tris·HCl, pH 8.0, from 0 to 0.75 M NaCl. The procedure for the isolation of Gla protein from swordfish and human bone and bovine dentine was altered only in the use of 50 mM rather than 5 mM NH_4HCO_3 to suppress Gla protein precipitation. Hydroxyapatite crystals were made by mixing calcium chloride and sodium phosphate solutions at 5 mM concentrations, pH 7.4, and 25°. Hydroxyapatite also was purchased from Clarkson Chemical Co. Amorphous calcium phosphate was prepared by mixing saturated solutions of calcium chloride and sodium phosphate at pH 7.4 and 25°.

Alkaline hydrolysis was carried out on 0.2–10 mg of protein

Abbreviations: Gla, γ-carboxyglutamic acid; Gla protein, γ-carboxyglutamic acid-containing protein.

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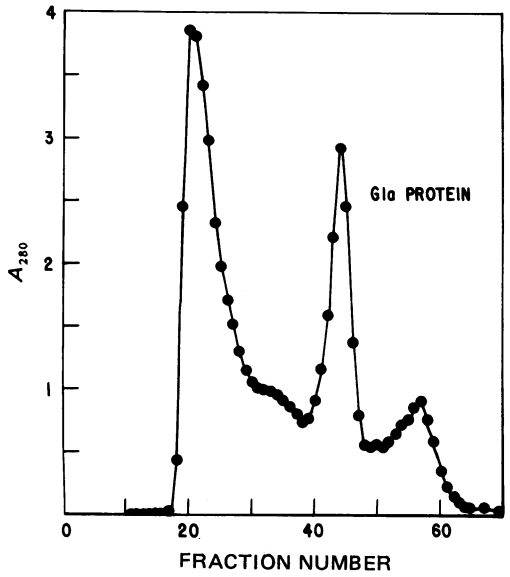

FIG. 1. Gel filtration of calf bone EDTA extract on a 2 × 100 cm column of Sephadex G-100 with 5 mM NH_4HCO_3 buffer, 25°, 4.3 ml per fraction.

in 4 M NaOH, 110°, for 24 hr as described by Hugli and Moore (10); starch was not used. Hydrolysates were diluted with 300 μl of 0.2 M citrate, pH 0.7, and titrated to pH 0.7 with 6 M HCl; final volumes were 1.1–1.2 ml. Alkaline hydrolysis of 20–100 mg of calcified tissue left a calcium phosphate precipitate that was removed prior to titration to pH 0.7. Samples (300 μl) were injected into the Beckman model 119 amino-acid analyzer. The column (0.9 × 43 cm) contained Beckman AA-15 resin. Color factors were determined for Gla by comparison of color yields of Gla with that of glutamic acid formed from Gla after 24 hr at 110° 6 M HCl; alanine was used as an internal standard. Methylcellosolve (Pierce) was used as the ninhydrin solvent. The synthesis of Gla was verified by mass, nuclear magnetic resonance, and infrared spectroscopy. The 15-residue amino-terminal sequence was determined with a Beckman Sequencer, model 890B, and residues were identified by gas chromatography of the phenylthiohydantoin derivatives and by amino acid analysis of the residues released from phenylthiohydantoin derivatives by hydrolysis in HI (11).

RESULTS

The analytical method for Gla determination in proteins developed here is based on studies with synthetic Gla. Synthetic Gla is completely stable under conditions of alkaline protein hydrolysis, and no destruction of Gla could be detected after 24 hr at 4 M NaOH and 110°. Gla emerges from the amino-acid analyzer column at 19 min, compared to 14 min for cysteic acid and 27 min for aspartic acid. Phosphoserine emerges close to the position of Gla but does not interfere with the analysis because it is quantitatively destroyed on alkaline hydrolysis. The ninhydrin color factor for Gla is unusually low, about 25% of the color factor for glutamic acid. A 3-fold increase in the ninhydrin coil dwell time gave no apparent increase in the Gla color factor. Alkaline hydrolysis and amino acid analysis of human prothrombin gave a peak in the same elution position as synthetic Gla. With the above color factor, the area of this peak corresponds to eight Gla residues per molecule, in agreement with the 10 Gla residues in bovine prothrombin (3).

Alkaline hydrolysis of calf bone gave a peak on the amino-acid analyzer that cochromatographed with synthetic Gla. Several observations indicate that this amino acid is in fact Gla. The unknown amino acid was first isolated from an alkaline hydrolysate of the purified, Gla-containing calf bone protein by ion exchange chromatography on a 56-cm column with Beckman AA-15 resin. This unknown amino acid and synthetic Gla were both converted to glutamic acid after 24 hr in 6 M HCl at 110°. The unknown amino acid from bone has the same low ninhydrin color factor and the same electrophoretic mobility at pH 6.4 as synthetic Gla. The unknown amino acid was also *N*-acetylated and perethylated (2), and its mass spectrum was determined on an LKB 9000 combined gas chromatograph-mass spectrometer. The mass spectrum of the unknown amino acid is the same as that for synthetic Gla derivatized in this way, and is consistent with the previously published mass spectrum of a different Gla derivative (8).

Table 1. Purification of Gla protein from calf bone

Purification step	Gla protein (mg/100 g of bone)	Gla/Glu*
Ground calf bone	370†	<0.01
EDTA solubilized proteins	260†	0.16
Sephadex G-100	249	0.51
DEAE-Sephadex A25	174	0.56

* Gla to Glu ratio determined from amino acid analysis of alkaline hydrolysates.

† Estimated from the quantity of Gla in the sample and the Gla content of the purified protein.

Purification of the Gla-containing protein from calf bone was monitored by the presence of Gla in alkaline hydrolysates at each stage (Table 1). About 70% of the Gla initially in calf bone is extracted in the first 8 days of EDTA dialysis at 4°. The remaining Gla can be extracted by grinding the bone to a smaller size and dialyzing against EDTA further; the collagen that remains is free of Gla. The Gla protein can be purified by filtration over Sephadex G-100 (Fig. 1) and chromatography over DEAE-Sephadex. Over 90% of the Gla in the EDTA extract can be recovered in the second protein peak to emerge from the Sephadex G-100 column, and this fraction is about 30% of the total protein in the extract. Purification of the protein can be followed by acrylamide gel electrophoresis at pH 8, inasmuch as the Gla protein migrates well ahead of all other proteins in the EDTA extract. After DEAE-Sephadex chromatography the Gla protein gives a single band on the acrylamide gel electrophoresis system and a single band on sodium dodecyl sulfate gel electrophoresis. It also appears in a sharp band at pH 3.75 upon isoelectric focusing in acrylamide gels.

The purified Gla protein from calf bone is a low-molecular-weight protein with an unusual amino acid composition. Sedimentation equilibrium measurements give a molecular weight of 6800 in 0.1 M NH_4HCO_3, assuming a $\bar{V}\rho$ of 0.75. The molecular weight estimate from sodium dodecyl sulfate gel electrophoresis is 7000–8500. The protein has three residues of Gla per molecule, as estimated from the peak at 19 min in the analysis of the alkaline hydrolysate (Table 2) or from the increase of 5 to 8 in glutamic acid recovery between alkaline and acid hydrolysates. It has one disulfide bond and no free sulfhydryl groups, as determined by carboxymethylation in 8 M urea with and without mercaptoethanol. Fourteen of the first 15 residues in the amino-terminal sequence have been identified (Fig. 2). Residue 9 is not one of the 20 common amino acids and not Gla; at present, we cannot identify this amino acid.

Table 2. Amino acid composition of Gla protein from bovine bone

Amino acid	Residues per 6800 molecular weight	Residues per molecule
γ-Carboxyglutamic acid*	2.9	3
Aspartic acid	8.0	8
Threonine	0.1–0.3	0
Serine	1.0	1
Glutamic acid*	5.2	5
Proline	6.1	6
Glycine	5.4	5
Alanine	5.2	5
Half-cystine	1.9	2
Valine	2.1	2
Methionine	0.0	0
Isoleucine	1.1	1
Leucine	6.1	6
Tyrosine	3.9	4
Phenylalanine	2.1	2
Histidine	1.8	2
Lysine	1.9	2
Arginine	3.9	4
Tryptophan†	1.0	1
Hydroxyproline‡	0.0	0
Total residues		59

* Determined from alkaline hydrolysis.
† Determined from alkaline hydrolysis (10).
‡ Determined spectrophotometrically.

Table 3. Distribution of γ-carboxyglutamic acid in calcified tissues

Sample	nmol Gla/ 100 mg sample
Cortical bone (femur)	
Calf (6 months)	158
Steer (2 years)	53
Cow (15 years)	65
Calf cancellous bone (femur)	154
Calf epiphyseal growth cartilage (femur)	<5
Steer tooth enamel	<5
Steer tooth dentine	26
Dogfish shark calcified cartilage (jaw)	<5
Swordfish vertebrae	119
Halibut vertebrae	126
Chicken cortical bone (femur)	39
Human cortical bone (tibia)	88

The presence of a Gla protein in many calcified tissues is indicated by the abundance of Gla in alkaline hydrolysates of these tissues (Table 3). To investigate the distribution of the Gla protein further, we also have purified a Gla protein from bovine dentine, swordfish vertebrae, and human cortical bone by procedures analogous to those used for calf bone. In each case the isolated Gla protein has approximately the same molecular weight, electrophoretic mobility, and Gla content. The Gla protein has also been purified from the cortical bone of a 15-year-old cow, and the protein has an amino acid composition identical to the calf bone protein.

The Gla protein binds strongly and reversibly to hydroxyapatite, and half of the protein is adsorbed at a crystal concentration of 0.4 mg/ml (Fig. 3). The Gla protein will not bind to amorphous calcium phosphate, a less ordered crystal form (12) which precipitates when the initial concentration of calcium and phosphate greatly exceeds the solubility product. Amorphous calcium phosphate is slowly converted to hydroxyapatite on standing, and this hydroxyapatite then binds the Gla protein as strongly as commercial hydroxyapatite or hydroxyapatite crystallized from slightly supersaturated solutions. The Gla protein is probably not associated with the collagen in bone. No binding of Gla protein to collagen could be detected with a 1000-fold weight excess of extracted, Gla-free bone collagen at pH 7.4 and 0.16 M KCl. The Gla protein also failed to associate hydroxyapatite crystals with demineralized, Gla-free bone collagen particles that had been passed through a 125-μm sieve. An initial covalent association of the Gla protein to the collagen matrix and its release by proteolytic cleavage is unlikely, inasmuch as the Gla protein can also be extracted quantitatively with 0.6 M HCl.

Because the Gla protein binds to hydroxyapatite and not to amorphous calcium phosphate, the protein might be expected to bind calcium and phosphate in the proper orientation to nucleate hydroxyapatite crystallization. However, the Gla protein reproducibly inhibits hydroxyapatite crystallization (Fig. 4). This inhibition is not caused by the net reduction in calcium concentration due to calcium binding to Gla residues in the Gla protein. Even if all three of the Gla residues were saturated with calcium, the free calcium concentration would be reduced by less than 1%, an amount too small to affect the rate of crystallization. In a series of experiments we find that the length of time before crystals appear increases in proportion to the increase in amount of Gla protein present. At a fixed concentration of Gla protein the length of time before crystals appear decreases with increased degree of supersaturation. Both of these observations support the idea that the Gla protein inhibits crystallization by combining with the initial crystal nuclei. Inasmuch as initial calcium and phosphate ion products range from 2.5 to 250 mM^2 in these experiments, the first crystals to form are hydroxyapatite and not amorphous calcium phosphate. The final concentration of Gla protein remaining in solution after crystallization is complete is the amount that would be bound at equilibrium to the hydroxyapatite present.

DISCUSSION

The Gla bone protein described here is a unique protein and is not related to the Gla-containing blood clotting factors. The possibility of the presence of blood clotting protein fragments in bone must be clearly ruled out, because the blood clotting factors that contain Gla also bind strongly to hydroxyapatite. Sequence analysis provides the strongest evidence for the uniqueness of the Gla bone protein. Three of the amino acids in the amino-terminal 15 residues of the bovine Gla bone protein, tyrosine, histidine, and tryptophan, are not found in the amino-terminal 40 residues of bovine prothrombin or bovine

Tyr-Leu-Asp-His-Trp-Leu-Gly-Ala-X-Ala-Pro-Tyr-Pro-Asp-Pro-
5 10 15

FIG. 2. Primary structure of the NH_2-terminal 15 residues of calf bone Gla protein.

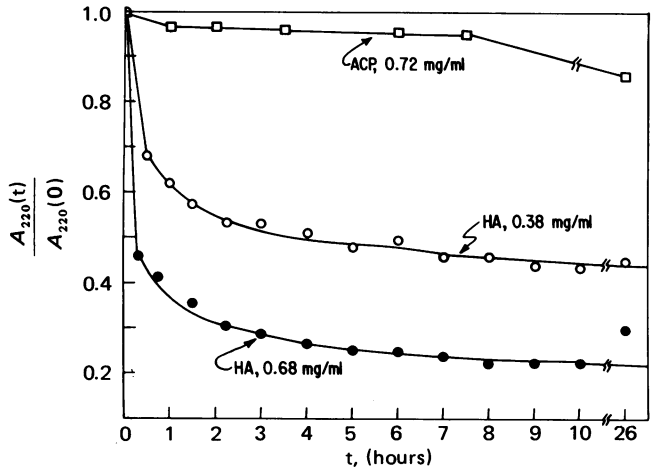

FIG. 3. Gla protein binding to hydroxyapatite. A small aliquot of concentrated Gla protein was added at zero time to a solution in equilibrium with the given crystal concentration. Solutions were stirred continuously at 25°. Initial protein concentration, 0.05 mg/ml; 5 mM Pipes buffer (1,4-piperazinediethanesulfonic acid) at pH 7.4; 25°; ionic strength adjusted to 0.16 with KCl. HA, hydroxyapatite; ACP, amorphous calcium phosphate.

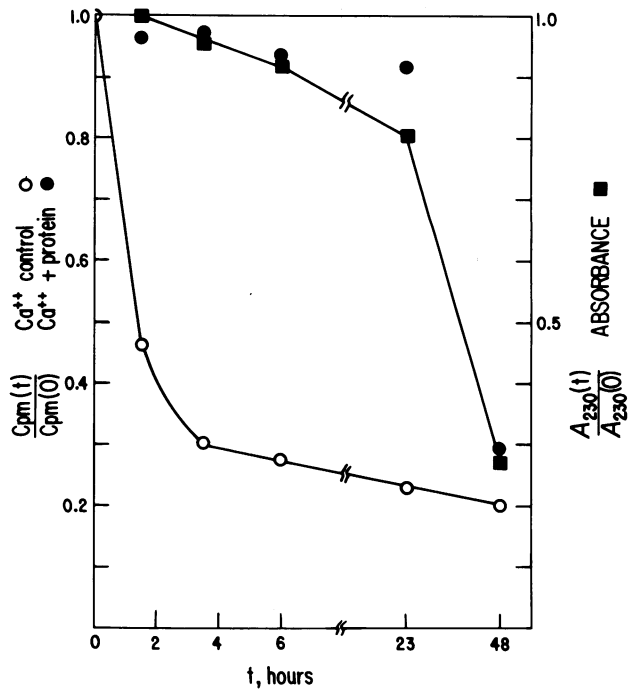

FIG. 4. Inhibition of crystallization. Equal volumes of $^{45}CaCl_2$-containing Gla protein and sodium phosphate, pH 7.4, were mixed at zero time to give initial concentrations of 5 mM in Ca^{2+} and phosphate ion and 0.08 mg/ml in Gla protein. Solutions contained 5 mM Pipes at pH 7.4; ionic strength was adjusted to 0.16 M with KCl, 25°. Radioactivity measurements and absorbance readings were taken on the supernatant after centrifugation. ○, No Gla protein; ● and ■, Gla protein.

factor X (3, 13). Also, no Gla residues are found in the amino-terminal 15 residues of the Gla bone protein, whereas three Gla residues are found in this region of prothrombin (3).

The calf cortical bone protein is representative of a class of closely similar and possibly identical proteins that are present in most bovine calcified tissues. We find that the Gla proteins from bovine cancellous bone and tooth dentine have approximately the same molecular weight, electrophoretic mobility, and Gla content as the cortical bone protein does. Further characterization will be needed to see if these proteins are identical. The only calcified tissue that does not have the Gla protein is tooth enamel, which also differs from the other calcified tissues in its lack of collagen. Because high quantities of the Gla bone protein are found in all ages of the cow, it seems likely that the protein is important to the steady-state function of calcified tissues.

All vertebrate bones tested appear to have large quantities of a Gla-containing protein with a low molecular weight and high electrophoretic mobility. We have purified the protein from bovine, swordfish, and human bone, and have detected Gla in alkaline hydrolysates of halibut and chicken bone. A similar Gla-containing protein component has also been recently isolated from chicken bone (9). The low molecular weight and the high electrophoretic mobility of these Gla proteins indicate that they are probably the major component of the anionic protein fraction previously isolated from EDTA extracts of fetal calf and chicken bones (14). We have been unable to find this protein or any Gla in the calcified cartilage of elasmobranches, which suggests that the Gla protein evolved in calcified tissues at the same time as the first true bone did. The presence of this unique Gla-containing protein in most calcified tissues and in bony vertebrates from fish to mammals suggests that the Gla protein must have an important function in calcified tissues.

Knowledge of the precise location of the Gla protein in calcified tissues is also important for an understanding of its possible functions. We have presented evidence that the Gla protein binds strongly to hydroxyapatite crystals, and we have presented evidence against either covalent or noncovalent association of the protein with bone collagen. The strength of binding of Gla protein to hydroxyapatite indicates that less than 0.01% of the protein would not be bound to the mineral phase in bone. The protein is 1–2% of the total protein in bone, and each 100 g of bone will contain from 3 to 6×10^{-5} mol of the protein. If an average crystal size of $45 \times 45 \times 400$ Å is assumed, each crystal in bone will have an average of 0.6–1.2 molecules of Gla protein bound to it. The absence of Gla protein binding to amorphous calcium phosphate suggests that the Gla protein is designed to bind to the specific surface structure of hydroxyapatite. It seems likely that this binding occurs partly through the association of the Gla side chain with Ca^{2+} atoms on the crystal surface, in analogy to the mode of prothrombin binding to mineral surfaces.

A possible function of the Gla protein could be in the regulation of mineralization in calcified tissues. We have shown that the Gla protein is a potent inhibitor of hydroxyapatite crystallization at physiological pH and ionic strength. Crystallization is delayed by more than a day at a Gla protein concentration only 2% of the concentration in bone and a calcium phosphate ion product 10 times the value in blood serum. At the higher *in vivo* concentration of Gla protein and the lower physiological ion product, crystallization would be delayed for much longer than a day. The inhibition of crystallization probably occurs through the association of Gla protein with the initial crystal nuclei. Analogous cases are the inhibition of calcium sulfate crystallization by polyglutamic and polyaspartic acids (15) and the inhibition of hydroxyapatite crystallization by polyphosphonates and polyphosphates (16). One function of a nucleation inhibitor in bone could be to suppress the spontaneous nucleation of crystals and thereby favor the growth of a few large crystals rather than of many small ones. A nucleation inhibitor

might also regulate the overall rate of mineral deposition in calcified tissues by reducing the number of crystal nuclei for growth. Although it is possible that the inhibition of hydroxyapatite crystallization by the Gla bone protein is merely the product of its binding affinity for hydroxyapatite and not related to its function, these experiments show that the Gla protein is definitely not a nucleating agent for hydroxyapatite crystallization as suggested previously (7).

Another possible function of the Gla protein could be to signal a loss of mineral to surrounding tissues. The Gla protein is bound exclusively to the mineral phase, and demineralization of a region of bone would release it at a high local concentration. This function leads to the prediction that the Gla protein should have a direct effect on cellular systems. Urist *et al.* have postulated the existence of a bone morphogenic protein in a series of studies on the factors that enable demineralized bone to induce new bone growth (17). Although the bone morphogenic protein is thought to be released from bone by the action of a protease (17) and not by desorption from hydroxyapatite, the bone morphogenic property provides a stimulating example of a tissue response to a noncollagenous bone protein signal.

The Gla protein could be a key to the understanding of pathological calcification of soft tissue. The calcification of arteries with age, or hardening of the arteries, results in hard lesions of hydroxyapatite crystal deposition. We find Gla in these hardened parts of human arteries at concentrations up to 60% of human bone. In preliminary experiments we have also extracted calcified aorta with EDTA and have isolated a protein with a similar low molecular weight and Gla content to the class of proteins described here. The presence of the Gla protein in such abundance in calcified arteries suggests that this protein may be involved in this and other pathological soft tissue calcifications.

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