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Direct identification of the calcium-binding amino acid, γ-carboxyglutamate, in mineralized tissue

(prothrombin/vitamin K/bone/barium sulfate/alkaline hydrolysis)

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ABSTRACT **A direct approach has been developed for quantitative identification of the calcium-binding amino acid, γ-carboxyglutamate, in proteins. This should be advantageous for the study of numerous systems where specific roles for the binding of calcium or other divalent cations are suspected. Investigation of mineralized tissue, where calcium-binding proteins are implicated in the mineralization process, revealed that γ-carboxyglutamate was present in proteins solubilized from chicken bone with neutral aqueous ethylenediamine tetraacetic acid. This was established by direct isolation of the amino acid from alkaline hydrolysates and its quantitative conversion to glutamic acid by decarboxylation in 0.05 M HCl at 100°. The kinetics of decarboxylation and chromatographic behavior are identical to those of γ-carboxyglutamate from human prothrombin. After resolution of the soluble bone proteins by phosphate gradient elution from hydroxyapatite, γ-carboxyglutamate was found to be concentrated primarily in one $BaSO_4$-adsorbable anionic protein species; bone collagen was devoid of the amino acid. In view of the recently discovered requirement of vitamin K for generation of calcium binding sites (γ-carboxyglutamate) by γ-carboxylation of specific glutamic acid residues in prothrombin, our findings may implicate vitamin K metabolism in normal bone development and suggest a role for the γ-carboxyglutamate-rich protein in regulation of calcium salt deposition in mineralized tissues.**

Calcium is ubiquitous in biological systems and has been implicated as an essential component in such diverse processes as muscle contraction, cell motility, membrane adhesion, synaptic transmission, and hormone release (ref. 1, and references contained therein). While sites for calcium binding are implicit in these and other calcium-regulated processes, detailed characterization of such binding sites is in an embryonic state. An elegant system for metabolic control of calcium binding has recently been elucidated for the vitamin K-dependent blood clotting factors (prothrombin and Factors VII, IX, and X). Through a post-translational enzymatic process with absolute requirements for vitamin K and bicarbonate ion (2), specific glutamic acid residues in prothrombin are γ-carboxylated (3–5). The resultant γ-carboxyglutamic acid (Gla) residues act as calcium-binding sites (6–9) and are essential for the normal calcium- and phospholipid-dependent activation of hemostasis (6, 8, 10). The structure of this unique calcium-binding amino acid has been recently established by mass spectrometry (3–5; Fig. 1). If the appropriate glutamic acid sites are not carboxylated, as occurs during vitamin K deficiency or antagonism with warfarin or dicoumarol-type drugs, then the resultant unmodified protein exhibits only weak, relatively nonspecific interaction with calcium ions (6, 11). In other proteins that interact with calcium, the divalent ions are bound at a variety of sites, most of which are rich in carboxyl side chains, such as in thermolysin (12), carp parvalbumin (13), and possibly in calsequestrin (14) and a vitamin D-dependent calcium-binding protein (15). Conformation of the peptide backbone is also an important feature in some calcium-binding sites (13, 16).

Abbreviation: Gla, γ-carboxyglutamate.

Because of the apparent affinity of Gla residues for calcium ions, there is some incentive for examining any protein that interacts with calcium for the presence of Gla. While this suggestion has been made previously (5, 17), the methods for detecting Gla are cumbersome and generally presuppose detailed knowledge of the protein under study. Gla is readily decarboxylated to glutamic acid by the strongly acidic conditions generally used for protein hydrolysis (3–5, 17). Thus it was necessary to use specific enzymatic digestion and elaborate purification in order to obtain the prothrombin oligopeptides in which the presence of Gla could be rigorously established by mass spectrometry (3–5). Reductive modification of Gla with [^{3}H]diborane allows isolation of a stable labeled derivative from acid hydrolysates (17), but this method is tedious, nonquantitative, and not particularly sensitive.

We have developed a simple, alternative procedure involving alkaline hydrolysis and amino-acid analysis for direct determination of Gla residues in modified proteins. The validity of the method was established with a Gla-containing model protein, human prothrombin. With this method we investigated the proteins of mineralized tissue, where there was reason to suspect that calcium-binding proteins play a crucial role in the regulation of calcium salt deposition (18). An attractive mechanism and possible control point for regulating calcification could be the post-translational carboxylation of glutamic (or aspartic) acid residues, particularly since these acidic residues are abundant in the EDTA-soluble organic matrix proteins of hard tissues and their carboxylation would have eluded detection by common acid hydrolysis techniques. This report describes the isolation of a protein component from EDTA extracts of chicken bone, which is richer in Gla even than human prothrombin. The role of this protein in regulating mineralization in bone and other hard tissues is under investigation.

MATERIALS AND METHODS

Protein (0.3–5 mg) for alkaline hydrolysis was suspended in 2 M KOH in alkali-resistant glass tubes (Corning no. 7280) and hydrolyzed at 106° for 22 hr under N_2. Samples were chilled and diluted, and 60 mg of dry $KHCO_3$ was added to buffer the end point. Adjustment to pH 7 with 70% $HClO_4$ was followed by centrifugation. Gla was found to be stable (<10% decarboxylation) for at least 6 months at −20° in alkaline hydrolysates of human prothrombin. Protein was hydrolyzed in 6 M HCl at 108° for 24 hr under N_2. A Beck-

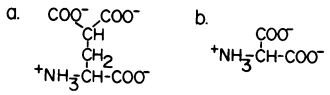

FIG. 1. Structure of (a) γ-carboxyglutamic acid (Gla) and (b) aminomalonic acid.

man/Spinco model 121 M amino-acid analyzer was used. The column (0.28 × 33.0 cm) contained Beckman AA-20 resin and was operated at 51° with a stepped series of 0.2 M citrate buffers ranging from pH 3.10 (0.16 M Na^+) to pH 7.13 (1.0 M Na^+). Gla was determined in alkaline hydrolysates using the ninhydrin color factor for glutamic acid. Other amino acids were measured in acid hydrolysates by standard techniques. No corrections were made for amino-acid destruction during hydrolysis.

Metatarsal bones of 14-week-old white Leghorn chickens were fragmented and scraped free of marrow, thoroughly washed in saline, pulverized in a mill cooled by liquid N_2, and then extracted twice with 0.5 M EDTA, pH 7.4 (19). The extracts were dialyzed exhaustively against distilled H_2O and then lyophilized. Hydroxyapatite chromatography was performed at 23° with a column (0.9 × 20 cm) of Hypatite C (Clarkson Chem. Co., Williamsport, Pa.). The EDTA-extracted protein was applied in 50- to 100-mg quantities in 1 mM phosphate buffer (KH_2PO_4–NaOH, pH 7.0) and eluted with a linear gradient of 1 mM–0.6 M phosphate. $BaSO_4$ adsorption of the EDTA-soluble proteins was done in the presence of 0.01 M oxalate at pH 7.0 (20). $BaSO_4$ adsorption of amino acids directly from alkaline hydrolyzates at pH 7.0 was useful for bulk isolation of Gla (5–25% yield) and provided up to 50-fold enrichment of Gla relative to glutamic acid. After washing with water, adsorbed amino acids were eluted from the insoluble $BaSO_4$ with 0.05 M HCl. Aminomalonic acid, an analog of Gla, was prepared as the barium salt from diethylacetamidomalonate (Aldrich) by hydrolysis in 2 M KOH (100°, 3.5 hr). On a molar basis the ninhydrin color yield of aminomalonate is only 40% of that for glycine. Normal human prothrombin containing Gla was kindly donated by Dr. Robert Rosenberg (Beth Israel Hospital, Boston, Mass.). This preparation was about 85% pure, containing small amounts of Factors IX and X. Proteins that were hydrolyzed to check for the general occurrence of Gla were of the highest available purity from Worthington, Inc. Thermolysin was obtained from Calbiochem. Disc gel electrophoresis was performed according to standard techniques (21, 22). Slab gel electrophoresis was done in 0.8% agarose made up and run in 0.05 M veronal buffer, pH 8.6, containing either 2 mM calcium lactate or 5 mM EDTA (23).

RESULTS

Aminomalonic acid was prepared as a convenient analog of Gla (Fig. 1) in order to gain some insight into the properties of this class of compounds. Aminomalonate elutes from the analyzer as a single ninhydrin-positive peak at 16.5 min, or about 2.5 min after cysteic acid and 2 min before Gla. Decarboxylation of aminomalonate occurs readily in acidic solutions (analogous to Gla), with glycine being the product formed. This reaction was quantitated both by the disappearance of aminomalonate and by the appearance of glycine. In 0.05 M HCl the decarboxylation is a simple first-order process with a half-time of 120 hr at 23°, 2 hr at 50°, and 1.5 min at 93° (Fig. 2).

The elution position of Gla on the analyzer was established with alkaline hydrolysates of human prothrombin

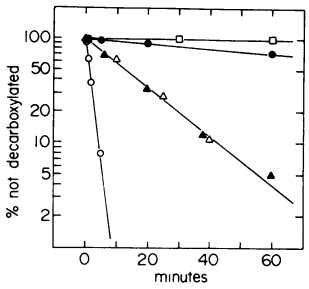

FIG. 2. Kinetics of decarboxylation in 0.05 M HCl: aminomalonic acid at 20° (□), 50° (●), and 93° (○); Gla at 100° from alkaline hydrolysates of human prothrombin (△) and EDTA-soluble chicken bone protein (▲).

which contained a significant ninhydrin-positive peak eluting 4.5 min after cysteic acid and 17 min before aspartic acid (Fig. 3). This very acidic compound, presumably Gla, was not found in acid hydrolysates of prothrombin as expected from the acid lability of Gla. When the alkaline hydrolysate was dissolved in 0.05 M HCl and heated at 100° for various times, the presumptive Gla peak disappeared with a half-time of 13 min (Figs. 2 and 3). Because of the large amount of glutamic acid in the alkaline hydrolyzate (15 times greater than Gla) it was not possible to measure accurately the increase in glutamic acid caused by Gla decarboxylation. Using the ninhydrin color factor for glutamic acid, a Gla concentration of 8.6 ± 0.5 residues per 1000 amino acid residues was calculated for the alkaline hydrolyzed prothrombin. If the true Gla content of human prothrombin is analogous to the 17.2 residues per 1000 residues in normal bovine prothrombin (5, 10, 11), then the yield of Gla by the present hydrolysis procedure is about 50%. The total amino acid composition of human prothrombin was in close agreement with published values for bovine prothrombin (10, 11).

Various protein fractions isolated from bone were subjected to alkaline hydrolysis and surveyed for Gla by amino acid analysis. The EDTA-soluble proteins of adult chicken bone showed a significant Gla peak that was not present in acid hydrolysates. The 570 nm/440 nm ninhydrin ratio was about 9.5, as was found for the Gla peak from prothrombin; the ratio for glutamic acid is 10.4. Concentration of the putative Gla was 4.3 and 8.3 ± 0.5 residues per 1000 residues in the first and second EDTA extracts, respectively (Table 1). No Gla (<0.3 residue per 1000 residues) was detected in alkaline hydrolysates of a variety of other materials, including bone matrix after thorough demineralization with

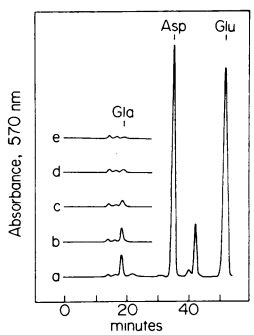

FIG. 3. Ninhydrin profile of alkaline hydrolyzed human prothrombin (a). Hydrolysate heated in 0.05 M HCl at 100° for 10 min (b), 25 min (c), 40 min (d), and 85 min (e), showing destruction of Gla peak.

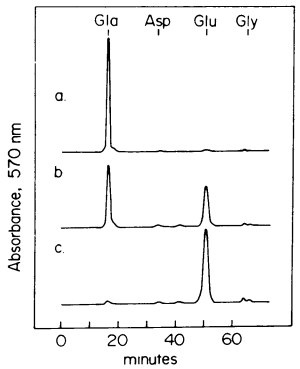

FIG. 4. Ninhydrin profile of Gla isolated from the EDTA-soluble protein of adult chicken bone (a); Gla heated 6 hr at 120° in pH 2.8 citrate (b); Gla heated in 3 M HCl at 120° for 6 hr (c).

EDTA, rat tail tendon collagen, egg-white lysozyme, α-lactalbumin, α-chymotrypsin, pepsin, bacterial collagenase, hyaluronidase, thermolysin, and pure EDTA; or any of the Gla-negative proteins with EDTA added before hydrolysis.

In order to provide further characterization of the presumptive Gla peak in the EDTA-soluble protein, we isolated the suspected ninhydrin peak from 25 mg of alkaline hydrolyzed protein by ion-exchange chromatography on Dowex-50 (0.6 × 150 cm column, 0.2 M citrate buffer, pH 2.8, 60°). Rechromatography of the isolated peak on the analyzer showed a single sharp component at the position of Gla with less than 2% contamination by other ninhydrin-positive materials (Fig. 4a). Merely heating the sample in the pH 2.8 citrate buffer for 6 hr at 120° before applying it to the analyzer column caused 50% destruction of the putative Gla with appearance of an equally large peak of glutamic acid (Fig. 4b). Hydrolysis of the putative Gla in 3 M HCl for 6 hr at 120° destroyed virtually all the parent compound with an apparently quantitative conversion to glutamic acid (Fig. 4c).

Identification of the Gla peak in alkaline hydrolysates of the chicken bone EDTA extract was corroborated by kinetic studies of the decarboxylation reaction. Gla was isolated free of interfering components by direct $BaSO_4$ adsorption of the alkaline hydrolyzate. The 0.05 M HCl eluate contained nearly equal concentrations of Gla (16.8 nmol/ml) and glutamic acid (16.0 nmol/ml), in addition to aspartic acid (73.2 nmol/ml). This solution was heated at 100°, and at various times aliquots were withdrawn, cooled, and subjected to amino acid analysis. Destruction of the Gla peak was accompanied by a corresponding increase in glutamic acid; aspartic acid was unchanged. The average ratio of moles of Gla destroyed per mole of glutamic acid produced was 0.83. Since the Gla concentration was calculated using the glutamic acid ninhydrin color factor, this ratio may differ from 1.0 because of an intrinsic difference in the color factors for the two compounds (as observed for aminomalonic acid and glycine). The kinetics of Gla decarboxylation are first-order, and the half-time is 13 min, in exact agreement with the value determined for the prothrombin hydrolysate (Fig. 2).

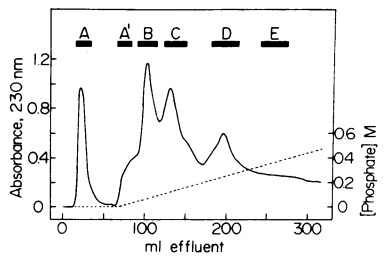

FIG. 5. Hydroxyapatite chromatography of EDTA-soluble proteins from adult chicken bone (—) eluted by a phosphate gradient at pH 7.0 (- - - -) as described in *Materials and Methods*. Peaks A through E were pooled as shown.

Table 1. γ-Carboxyglutamic acid concentration in EDTA-soluble bone proteins

Fraction	Gla*	% γ-Carboxylation†
1st EDTA extract	4.3	3.2
2nd EDTA extract	8.3	5.3
Hydroxyapatite peaks		
A	<0.3	<0.4
A'	<0.7	<0.7
B	2.4	1.9
C	14.9	9.0
D	1.8	1.0
E	1.2	0.6
$BaSO_4$-adsorbed	13.8	7.2

* Expressed as residues per 1000 residues ± 0.5 SEM; not corrected for destruction; average of duplicate analyses on pools from two separate chromatographic runs.

† Equal to the ratio: 100 × Gla/(Gla + glutamic acid).

Chromatographic resolution of the EDTA-soluble proteins was pursued in order to identify the component(s) that contained Gla (Fig. 5). The various peaks were collected and analyzed for amino acid content by alkaline and acid hydrolysis (Table 1). Space considerations preclude presentation of complete amino acid compositions for all peaks. Collagen components of the EDTA extract elute from hydroxyapatite at very low phosphate concentrations, while the other proteins are retarded primarily according to their content of acidic amino acids. Gla is found in one of the peaks (C of Fig. 5) at 14.9 ± 0.5 residues per 1000 residues, which is about 1.7 times the level in the human prothrombin. This peak contains more than 60% of the total Gla present in the EDTA-soluble proteins, although it amounts to only about one-sixth of the total protein. Peak B contains about 15% of the total Gla (2.4 ± 0.5 residues per 1000 residues), with smaller amounts appearing in the other components. The two fractions with collagen-like composition A and A', are devoid of significant levels of Gla. The presence of hydroxyproline in peak C (7.9 residues per 1000 residues) suggested that some collagen-like material was present as a contaminant. Since proteins containing Gla are known to have unusual affinity for barium salts (6, 20), $BaSO_4$ adsorption was performed on the first EDTA extract. The adsorbed protein represented about 12% of the total protein and was similar in Gla composition to peak C (13.8 ± 0.5 residues per 1000 residues) yet devoid of hydroxyproline. Polyacrylamide gel electrophoresis showed that peak C material, which migrated as a single major band with minor contaminants, had the greatest anodal mobility at pH 8.9 of the principal components in the EDTA extract. In agreement with the increased Gla content of the second EDTA extract (Table 1), the electrophoresis band corresponding to peak C was enriched in this material relative to the first EDTA extract. Sodium dodecyl sulfate-polyacrylamide gel studies indicated a molecular weight of about 10,000–12,000 for both peak C and the $BaSO_4$ adsorbed protein. In agarose gels, peaks B and C have a significantly greater anodal mobility at pH 8.6 in the presence of EDTA than in the presence of calcium, suggesting

that these components have calcium-binding activity. Direct alkaline hydrolysis of saline-washed, undecalcified bone samples provided a means for estimating the amount of peak C protein in whole bone. The observed Gla content (0.22 and 0.26 ± 0.05 residue per 1000 residues in 16-day chick calvaria and adult chicken metatarsal, respectively) indicates that about 1% of the total bone protein at both ages may be attributed to peak C material.

DISCUSSION

The difficulty of quantitatively detecting Gla in uncharacterized proteins or in complex protein mixtures prompted our development of the present approach. This manner of coping with the problem should be of interest to others involved with the isolation and characterization of calcium-binding proteins. Identification of Gla by specific hydrolysis procedures, simple amino acid analysis, and by the kinetics and products of decarboxylation is feasible for milligram quantities of any protein where the presence of Gla is suspected. While it is obviously desirable to present incontrovertible proof of the structure of Gla in peptide linkage, as elegantly presented by others for prothrombin (3–5), this is technically impossible for many systems of interest.

Our analysis of the proteins of mineralized tissue focussed on Gla because calcium binding by proteins of the matrix has generally been proposed as an initial step in the mineralization process in both normal and pathologic states (16, 18). There is abundant evidence that mineral deposition is directed at the local level by macromolecular components of the organic matrix (18, 24). Calcium incorporation in mineralizing fetal rat bone is known to precede phosphate deposition by several hours when ^{45}Ca and $^{32}P_i$ are simultaneously injected (25). Nucleation of calcium phosphate crystallization by organic matrix constituents requires interaction of the component mineral ions with sites on the matrix to form ordered ion clusters (18). Collagen, the predominant protein in bone and dentin, can provide, in its 640 Å periodic fibrillar form, nucleation sites for hydroxyapatite deposition both *in vivo* and *in vitro* (18, 24). Acidic proteins and phosphoproteins of bone (19), dentin (26–28), and enamel (29), glycoproteins (19, 27, 30), and polar lipids (31) are integral constituents of mineralizing tissues and also have been implicated in the calcification process. The likely sites of calcium affinity are generally thought to be anionic side chains of organic matrix proteins. Phosphoserine residues (18, 26, 28, 29) and carboxyl groups of glutamic and aspartic acids (32) have been investigated in this regard. Demineralization of hard connective tissues with neutral EDTA solutions solubilizes a class of proteins rich in these acidic amino acids, and it has been suggested that these proteins are of critical importance to the mineralization process (19, 28).

We have established the presence of Gla in the EDTA-soluble proteins of chicken bone. The Gla is located primarily in one protein species (Table 1), with smaller amounts in other components. This protein, whether obtained by hydroxyapatite chromatography (peak C) or $BaSO_4$ adsorption, is about 70–80% pure, as judged by gel electrophoresis, with an estimated molecular weight of 10,000–12,000 for the primary component in sodium dodecyl sulfate-polyacrylamide gels. The protein is nondialyzable and exhibits a calcium-dependent electrophoretic mobility in agarose gels, suggestive of calcium-binding activity. About 9% of the glutamic acid residues in the Gla-rich protein are γ-carboxylated and occur as Gla. No Gla is found associated with the collagenous proteins of the EDTA extract or in the demineralized bone residue, but the possibility that Gla-containing polypeptides are covalently linked to bone collagen or procollagen *in vivo* cannot be ruled out. Although it is conceivable that the Gla-containing components of bone are "bone-seeking" polypeptides which are elaborated by proteolysis of the vitamin K-dependent clotting factors, several lines of evidence weigh heavily against this possibility. (*i*) The amino acid composition of the peak C and $BaSO_4$-adsorbed proteins are significantly different (lower Gla, Thr, ½ Cys, and Arg; higher Asp, Ala, Tyr, and His) from the amino-terminal peptides released by proteolysis of bovine prothrombin (10), if comparison across this species gap is valid. (*ii*) The abundance of peak C material in bone is high (≃1% of total bone protein) in both embryonic and adult chicken bone, which would provide as much as one molecule for every three or four tropocollagen molecules. (*iii*) The second EDTA extraction contains about twice as much Gla-rich protein as the first, suggesting an intimate relationship of this protein to the bone matrix rather than superficial adsorption to the mineral phase.

If biosynthesis of Gla residues occurs in the osseous tissue, then by analogy to the prothrombin system in liver (2) we might expect vitamin K-dependent incorporation of CO_2 into newly formed Gla in bone proteins. Preliminary studies with cultured chick calvaria have demonstrated that radioactivity from [^{14}C]bicarbonate is rapidly incorporated into the EDTA-soluble protein fraction. In alkaline hydrolyzates a ^{14}C-labeled, ninhydrin-positive peak with the mobility of authentic Gla is observed. This peak is destroyed by acid hydrolysis with resultant increases in the glutamic acid peak, in agreement with data for rat prothrombin carboxylated *in vitro* (2). If enzymatic carboxylation of the proteinaceous constituents of the bone matrix is a vitamin K-dependent process, then anomalies might be expected to occur in mineralized tissues under conditions of vitamin K deficiency or chronic antagonism of vitamin K by dicoumarol or warfarin anticoagulant therapy. Obviously, a normal blood clotting system is of far greater necessity to survival than a normal skeletal structure. Hence, many long-term effects of vitamin K perturbation on mineralized tissues may remain undiscovered because of preemptive fatality due to bleeding problems. Numerous case studies of women receiving dicoumarol-type anticoagulant therapy during the first trimester of pregnancy indicate an extremely high incidence of fetal bone abnormalities (33, 34).

Prothrombin is the first example of a protein in which specific calcium-binding sites (Gla) are generated enzymatically by a post-translational carboxylation reaction (2–5). Might this same mechanism operate in other proteins involved in such disparate processes as cell motility, membrane adhesion, secretion, mitosis, muscle contraction, or enzyme catalysis where interaction with calcium or other divalent cations is essential? Our demonstration that a Gla-rich protein exists in bone suggests that specific protein carboxylation (and possibly decarboxylation) may have an important role in regulating mineral deposition in calcified tissue. Assuming this to be the case, then aberrations in the carboxylation process could cause abnormalities in bone, dentin, and enamel, and may even induce pathological calcification of the soft connective tissues. The simple procedure that we report for the determination of Gla in proteins may provide a fulcrum for investigation of a host of biological processes involving calcium, as well as problems related to the development and remodeling of mineralized tissues.

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