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Immunochemical Studies of Conformational Alterations in Bone γ-Carboxyglutamic Acid Containing Protein†

Pierre D. Delmas,‡ Debra D. Stenner, Robert W. Romberg, B. Lawrence Riggs, and Kenneth G. Mann*

ABSTRACT: The Ca^{2+}-dependent transition of the vitamin K dependent bone protein bone Gla-containing protein (BGP) was investigated by use of anti-BGP antibody that reacts with the Ca^{2+}-dependent conformation of BGP. Antibody binding occurred in the presence of Ca^{2+} or Mg^{2+} with a K_d(app) of 1.75 mM for Ca^{2+}. Upon removal of Ca^{2+} with ethylenediaminetetraacetic acid, antibody binding was eliminated. Upon thermal acid decarboxylation of BGP, Ca^{2+}-independent binding of the antibody was restored. Thus, the epitope not expressed by fully carboxylated BGP in the absence of calcium ion was restored either by addition of Ca^{2+} or by decarboxylation of the protein. Circular dichroic studies of fully carboxylated and fully decarboxylated BGP indicated that addition of Ca^{2+} to the fully carboxylated protein or decarboxylation to produce the glutamic acid containing equivalent of BGP resulted in increased order structure (apparent α-helix) in the protein, and this alteration was coinicident with antibody binding. These data suggest that carboxylation of this vitamin K dependent protein may lead to increased disorder in the protein as compared to the glutamic acid containing equivalent. Upon Ca^{2+} binding a structure more equivalent to the Glu-containing protein is obtained.

Bone Gla-containing protein (BGP)[1] is a noncollagenous protein specific for bone tissue that contains three residues of the unique vitamin K dependent amino acid γ-carboxyglutamic acid (Gla) (Hauschka et al., 1975; Price et al., 1976). BGP binds to Ca^{2+} and more strongly to hydroxyapatite crystals (Hauschka et al., 1975; Price et al., 1976; Hauschka & Gallop, 1977; Poser & Price, 1979), and the presence of the three Gla residues is generally thought to be responsible for this Ca^{2+} interaction. The biological function of BGP in bone physiology is still unknown but might be related to its interaction with Ca^{2+} and hydroxyapatite.

Ca^{2+} mediates a structural transition in some of the vitamin K dependent blood-clotting proteins, including prothrombin (Nelsestuen, 1976; Stenflo, 1977; Bloom & Mann, 1978; Tuhy et al., 1979; Furie et al., 1979; Madar et al., 1980) and factor X (Keyt et al., 1982). Recently, a Ca^{2+}-dependent confor-

† From the Section of Hematology Research and the Division of Endocrinology/Metabolism and Internal Medicine, Mayo Clinic/Foundation, Rochester, Minnesota 55905. *Received October 26, 1983; revised manuscript received April 9, 1984.* This work was supported in part by Research Grants AM-27065 and HL-16150, U.S. Public Health Service, NIAMDD, a grant from the R. K. Mellon Foundation, and the Mayo Foundation.

‡ Present address: Clinique de Rhumatologie, Hopital E. Herriot, 69003 Lyon, France.

[1] Abbreviations: BGP, bone Gla-containing protein (also called osteocalcin); Gla, γ-carboxyglutamic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin.

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mational change that is diminished after decarboxylation of the Gla residues has been reported for chicken BGP (Hauschka & Carr, 1982).

We have used conformation-specific antiserum to BGP to study the conformational alteration that occurs in this protein upon Ca^{2+} binding and the relationship of the γ-carboxyglutamate to the conformation of the protein.

Experimental Procedures

Purification of BGP. BGP was extracted from bovine bone by ethylenediaminetetraacetic acid (EDTA) and purified by gel filtration on a Sephadex G-100 column and by ion-exchange chromatography on a DEAE-Sephadex A-25 column as previously described (Price et al., 1976). Its authenticity was assessed by amino acid composition, Gla-residue content, and the sequence of the first eight amino acids from the amino-terminal region of the molecule.

Decarboxylation of BGP. Purified bovine BGP was decarboxylated according to the method of Tuhy et al. (1979). Thermal decarboxylation was achieved during either 12 or 24 h. Amino acid analysis after alkaline hydrolysis was performed on a Beckman 119 amino acid analyzer and on a Waters high-performance liquid chromatograph using citrate buffers and *o*-phthalaldehyde as described by Hauschka (1977). As a positive control, an aliquot of BGP prior to decarboxylation was analyzed with each decarboxylated BGP sample. The degree of decarboxylation of each sample was determined by comparing the amount of Gla per molecule of decarboxylated BGP to the amount of Gla per molecule of fully carboxylated BGP as reported by Poser et al. (1980).

Antibody Binding Studies. The binding of BGP with antiserum was measured by using a double antibody radioimmunoassay system modified from that described by Price & Nishimoto (1980). The same sample of normal (carboxylated) BGP or decarboxylated BGP was used for standard and tracer. Radioiodination of carboxylated or decarboxylated BGP was performed by the chloramine-T method (Greenwood et al., 1963). Antiserum against fully carboxylated BGP was raised in rabbits as previously described by Price & Nishimoto (1980) (Delmas et al., 1983). The antiserum chosen for these binding studies (R102) was chosen on the basis of its Ca^{2+} dependence of binding and was used at a final dilution of 1:20 000. All assays contained (a) a known amount (from 0 to 10 ng) of unlabeled BGP in 0.1 mL of assay diluent, (b) primary antiserum (R102) added in 0.2 mL of a 1:40 dilution of normal rabbit serum in assay diluent, and (c) 10 000 cpm of ^{125}I-labeled BGP (carboxylated or decarboxylated) in 0.1 mL of assay diluent for a final incubation volume of 0.4 mL. The assay diluent contained 0.02 M tris(hydroxymethyl)aminomethane (Tris), 0.15 M NaCl, 1% bovine serum albumin (BSA), 1% Triton, pH 8.0, and either 2 mM Ca^{2+} or 25 mM EDTA. In binding studies performed at low ionic strength conditions, the assay buffer did not contain NaCl.

Assay mixtures were incubated in plastic test tubes 12 mm by 75 mm for 4 h at 37 °C. Rabbit antiserum was precipitated by adding two units of goat antiserum to rabbit γ-globulin (lot 160701, Calbiochem Behring Corp., San Diego, CA) in 0.1 mL of assay diluent. After the mixture was incubated for 14 h at 4 °C, the tubes were centrifuged to sediment ^{125}I-labeled BGP bound to the rabbit antibodies, and the supernatant was discarded. The pellet was washed with 1 mL of assay diluent and recentrifuged and the supernatant discarded. Nonspecific binding of ^{125}I-labeled BGP, measured by incubating ^{125}I-labeled BGP and normal rabbit serum, without specific antiserum, followed by the usual second antibody precipitation, is 2%. (The presence or absence of Ca^{2+} had no influence on this value.) Total and antibody-bound ^{125}I-labeled BGP were determined by counting in a Beckman 8000 γ counter for 1 min. The fraction of ^{125}I-labeled BGP bound to antiserum ("B") was defined as counts per minute in precipitate minus counts per minute in background divided by total counts per minute in assay; maximum binding ("B_0") was the value of B when no unlabeled BGP was present. B and B_0 values were the average of three independent determinations.

For the Ca^{2+} titration curve studying the binding of either carboxylated or decarboxylated labeled BGP to antiserum in the presence of increasing amounts of Ca^{2+}, all reagents used in the assay were dialyzed against 10 mM EDTA and then dialyzed extensively to remove EDTA. The binding (B_0) of this metal ion free BGP to antiserum was then studied by adding various amounts of Ca^{2+} (from 0 to 10 mM) to the assay buffer.

Circular Dichroism (CD). Circular dichroism spectra in the far-ultraviolet region (190–250 nm) were recorded with a Jasco Model J-20A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) at 25 °C with protein concentrations of 0.11–2.7 mg/mL in a 0.10-cm path-length cuvette. Spectra were recorded as the average of 16 scans with a Jasco DP-500N data processor. Base line deviations were subtracted from all spectra. The spectropolarimeter was standardized with recrystallized *d*-10-camphorsulfonic acid (Chen & Yang, 1977); an extinction coefficient of 35.6 M^{-1} cm^{-1} at 285 nm was used (Vallee & Holmquist, 1980).

Mean residue ellipticity $[\theta]$ was calculated from

$$[\theta]_\lambda = \theta_{obsd}(MRW)/(10dc)$$

where λ = wavelength, θ_{obsd} = observed ellipticity in degrees, MRW = mean residue weight, c = concentration in grams per milliliter, and d = path length in centimeters. Concentrations of BGP were calculated from an extinction coefficient of 1.06 (Poser & Price, 1979) for a 0.1% solution of protein at 280 nm, and the mean residue weight of 119.3 was calculated from a molecular weight of 5845 on the basis of the known sequence of calf bone BGP (Price et al., 1977).

All circular dichroic data were signal averaged on a DP-500 data processor using 16 repetitive scans. With five independent samples of BGP, the experimental error in ellipticity was calculated to be in the range of 7–10%. These errors would lead to experimental errors in the calculation of α-helix, β-pleated sheet, and random coil of approximately 2%.

Estimates of *apparent* α-helix, β-pleated sheet, and random coil were determined by the method of Chen et al. (1972) using a least-squares approximation computer program written with the help of Aloysius Chu. Since this method is based on only five proteins with known crystal structure as standards, the calculated percentages of α-helix, β-sheet, and random coil can only be taken as estimates of structure. The term "random coil" here is used in the context of circular dichroic measurements rather than inferring that true random coil, indeed, exists within proteins for which crystal structures have been determined. A better term perhaps would be "nonregular-ordered structure". The quantitation of various structural contributions to the secondary structure of proteins can only be deduced explicitly from X-ray crystal data.

The value of quantitative analysis of circular dichroic data by techniques such as that of Chen et al. (1972) is not so much to explicitly determine the contributions of various regular structures to a protein but rather to provide a quantitative means of comparing spectral data.

Sedimentation Equilibrium. Sedimentation equilibrium data were collected on a Beckman Model E analytical ultracentrifuge equipped with a photooptical scanner. The signal

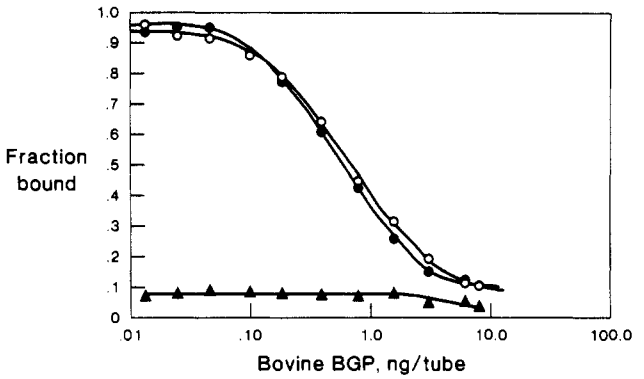

FIGURE 1: Divalent cation dependent binding of antibodies to bovine bone Gla protein. The relative fraction of radioactive bovine BGP bound is plotted vs. the quantity of competing cold antigen present in each tube. The closed circles indicate the presence of 2 mM $CaCl_2$, open circles the presence of 10 mM $MgCl_2$, and closed triangles the presence of 25 mM EDTA.

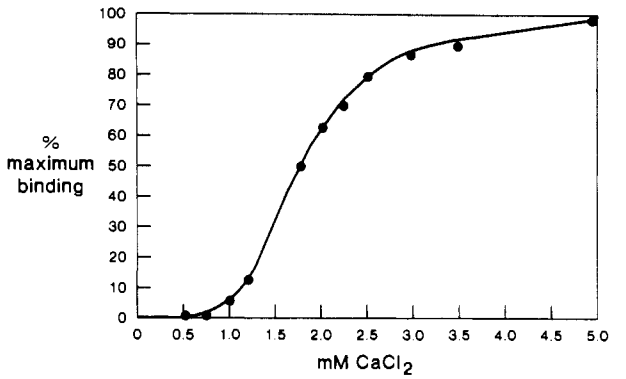

FIGURE 2: Ca^{2+} dependence of bovine BGP antibody binding. The fraction of ^{125}I-BGP bound to antibody in the presence of varying concentrations of $CaCl_2$ is plotted.

from the photooptical scanner was collected on an Apple IIE microcomputer by means of a Cyborg interface. Physical data were reduced by use of software developed in this laboratory. Sedimentation equilibrium experiments were conducted with BGP at an initial concentration of 0.84 mg/mL at a speed of 44 000 rpm.

Results

Metal-Dependent Binding of BGP to Antiserum R102. The binding of fully carboxylated BGP (≥95%) to different antisera in the presence of either Ca^{2+} or EDTA in the assay buffer was evaluated. Binding of BGP to antiserum R102 was found to be Ca^{2+} dependent as shown in Figure 1. In the presence of 2 mM Ca^{2+}, 43% of ^{125}I-BGP was bound to the antiserum R102 under conditions of antisera–antigen concentration chosen. This maximum binding (B_0) referred to as 100% on Figure 1 could be reduced to 0% by competition with unlabeled BGP. When the same binding experiment was performed with 25 mM EDTA instead of 2 mM Ca^{2+} in the assay buffer, there was minimal interaction between BGP and antiserum R102 as shown by the binding of ^{125}I-BGP in the presence or in the absence of the unlabeled BGP. Binding curves in the presence or the absence of Ca^{2+} were not modified by eliminating NaCl from the assay buffer (data not shown). Antibody–antigen complex formation could also occur in the presence of Mg^{2+}. As shown in Figure 1, in the presence of 10 mM $MgCl_2$ the maximum binding and the competition of unlabeled BGP with labeled BGP were nearly identical with the competition curve obtained with 2 mM Ca^{2+}.

Although unlikely, it was possible that the failure of antiserum R102 to bind to BGP in the presence of EDTA was not due to the absence of Ca^{2+} but to direct interference by EDTA in antibody–antigen complex formation. For this reason, BGP and all reagents used in the radioimmunoassay were rendered metal free ($\leq 10^{-7}$ M) and the effect of varying concentrations of Ca^{2+} on the interaction of the BGP–Ca^{2+}-specific antiserum with BGP was studied. The binding of BGP to the antiserum was determined as a function of Ca^{2+} concentration. In this experiment, ^{125}I-BGP, at trace levels, was incubated with antisera at the concentration used in the assay with varying concentrations of calcium ion. As shown in Figure 2, no binding was observed in the absence of Ca^{2+}; half-maximal binding (50%) was achieved with 1.75 mM Ca^{2+}. At concentrations above 5 mM, only small increases of the maximum binding could be detected.

Binding of Decarboxylated BGP in the Presence and in the Absence of Ca^{2+}. The binding of fully decarboxylated BGP

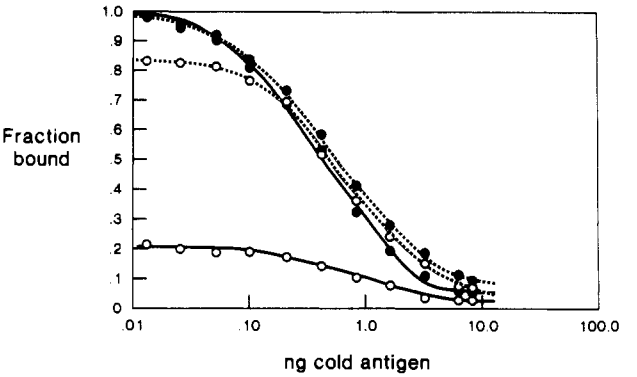

FIGURE 3: Comparison of the Ca^{2+} dependence of antibody binding to fully carboxylated and decarboxylated bovine BGP in the presence and absence of 2 mM $CaCl_2$. Data for the fully carboxylated protein in the presence of calcium ion are shown by the solid line with closed circles. Data for the fully decarboxylated protein in the presence of calcium ion are shown by the dotted line with closed circles. Data for the fully decarboxylated protein in the absence of calcium ion are shown by the dotted line with open circles and for the fully carboxylated protein in the absence of calcium ion by the solid line with open circles. For each of the four competition curves shown, the equivalent labeled and unlabeled competitors were used, i.e., fully decarboxylated with fully decarboxylated and fully carboxylated with fully carboxylated protein.

to antiserum R102 in the presence and absence of Ca^{2+} is shown in Figure 3. Following the decarboxylation treatment, BGP bound about 70% as well as the untreated protein in the presence of Ca^{2+}. In the absence of Ca^{2+}, the fully decarboxylated BGP binding was virtually equivalent to that observed in the presence of Ca^{2+}.

The competitive binding isotherms for fully decarboxylated and fully carboxylated BGP, as a function of the concentration of the respective unlabeled proteins, are shown in Figure 3. In this experiment, competition for labeled protein binding by the identical unlabeled proteins, i.e., decarboxylated BGP or carboxylated BGP, is represented. These competition curves are presented in the presence and absence of 2 mM Ca^{2+}. In the presence of Ca^{2+}, both carboxylated and decarboxylated BGP show similar titration curves, suggesting equal antibody affinity for both proteins. However, in the absence of Ca^{2+}, the binding isotherm of the decarboxylated BGP is almost unaltered while the binding isotherm for fully carboxylated BGP shows greatly reduced binding.

The coidentity of the Ca^{2+}-dependent epitope seen in fully carboxylated BGP with the epitope seen by the antibody in the decarboxylated protein was examined by a titration experiment in which the binding of radiolabeled ligands was subjected to competition with a single cold ligand. The data of Figure 4 illustrate a titration experiment in which radio-

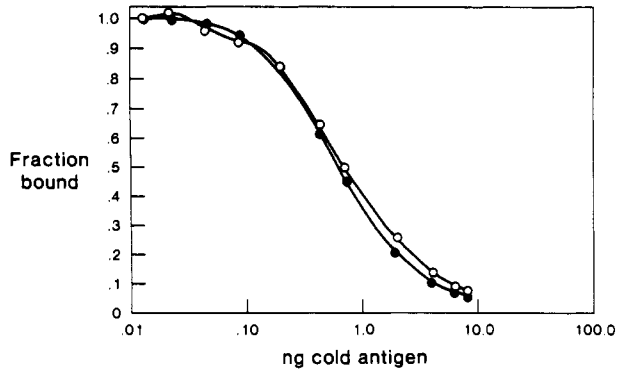

FIGURE 4: Competition of fully carboxylated and fully decarboxylated radiolabeled BGP by unlabeled fully carboxylated BGP. The fully carboxylated protein is seen in the closed circles; the fully decarboxylated radiolabeled protein is seen in the open circles.

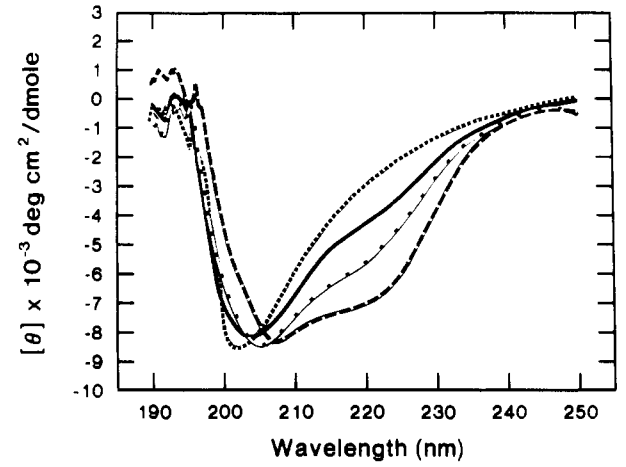

FIGURE 5: Circular dichroic spectra of fully carboxylated and decarboxylated BGP in the presence and absence of calcium ion: fully carboxylated protein in the presence of calcium ion (– –) and fully carboxylated protein in the absence of calcium ion (- - -); decarboxylated protein in the presence of calcium ion (■–■) and decarboxylated protein in the absence of calcium ion (—).

labeled carboxylated BGP and radiolabeled decarboxylated BGP are subjected to competition with unlabeled fully carboxylated cold BGP. The data indicate identical isotherms, which confirm the notion that the same epitope is being expressed in both carboxylated and decarboxylated proteins.

These data suggest that the Ca^{2+} dependence of the epitope(s) represented by the binding isotherms of fully carboxylated BGP is lost when BGP is decarboxylated. At the same time, the relative affinity of the antisera for the epitope is unaltered. These data suggest that Ca^{2+} binding by the fully carboxylated molecule results in a structure similar to that for fully decarboxylated BGP. Upon carboxylation of the appropriate glutamic acid residues in BGP, a structural transition occurs that results in loss of this epitope, and the structural loss is recovered upon Ca^{2+} binding. For the fully decarboxylated protein, the structure is unaltered by Ca^{2+} and is more representative of the Ca^{2+}–BGP structure.

Circular Dichroic Studies. In order to assess the gross features of the structures of fully carboxylated and decarboxylated BGP in the presence and absence of calcium ion, circular dichroic studies were performed to assess the implications of the immunochemical studies. BGP was analyzed over the wavelength region between 190 and 250 nm, and the spectra of BGP and decarboxylated BGP, with and without Ca^{2+}, are represented in Figure 5. Mean residue ellipticites were ±10% at 222 nm on the basis of five independently prepared samples. As predicted from the immunochemical

Table I: Apparent Quantitative Assessment of Secondary Structure in Bovine Bone Gla Protein[a]

	α-helix (%)	β-pleated sheet (%)	random coil (%)
BGP – Ca^{2+}	1 (8)[b]	20	79
BGP + Ca^{2+}	14 (35)[b]	20	67
dBGP[c] – Ca^{2+}	5 (18)[b]	21	74
dBGP[c] + Ca^{2+}	9 (26)[b]	21	70

[a] The precision was ±2% for α-helix, ±3% for β-pleated sheet, and ±2% for random coil. [b] Data for chicken BGP from Hauschka & Carr (1982) are given in parentheses. [c] 80% decarboxylated.

studies, the spectra of decarboxylated BGP, with and without Ca^{2+}, are similar and relatively more similar to the spectra of fully carboxylated BGP in the presence of Ca^{2+}. In the absence of Ca^{2+}, a significant qualitative shift in the circular dichroic spectra is observed for fully carboxylated BGP. The results of the circular dichroic study are consistent with the notion that decarboxylated BGP and fully carboxylated BGP in the presence of calcium ion are represented by more similar structures and that removal of calcium ion from the fully carboxylated protein results in a relatively extensive conformational alteration that is not manifested by the decarboxylated protein. In an attempt to provide a more quantitative assessment of the circular dichroic characteristics of these proteins, the spectra were analyzed by using the curve-fitting procedure of Chen, using the curve-fitting technique described under Experimental Procedures. These data are presented in Table I. As suggested by subjective evaluation of the shape of the circular dichroic spectra of Figure 5, quantitative assessment suggests a more ordered structure for the decarboxylated protein, with or without Ca^{2+}, and a greater similarity to the fully carboxylated protein in the presence of Ca^{2+}. Also in Table I, our data obtained for bovine BGP are compared with those of Hauschka & Carr (1982) for chicken BGP in the presence of Ca^{2+}.

The circular dichroic change observed upon Ca^{2+} binding is most probably associated with a change in the backbone structure of BGP. Aromatic amino acids have absorption maxima near 280 nm, and circular dichroic changes due to aromatic amino acid changes would be expected to be at wavelengths greater than 250 nm. Hauschka and Carr have measured a Ca^{2+}-dependent circular dichroic change in chicken BGP of -174 ± 4 cm^{-2} dmol^{-1} at 277 nm, which they attributed to tyrosine residues. The Ca^{2+}-dependent circular dichroic change due to aromatic amino acids is much smaller than that observed for the Ca^{2+}-dependent change in the far-UV, and the aromatic amino acid shift is too far red shifted to have a significant effect on the circular dichroic signals at wavelengths lower than 250 nm.

The circular dichroic spectra of BGP in the presence of Ca^{2+} were measured over a concentration range of 0.116–2.75 mg/mL with no apparent change in the circular dichroic spectra. The absence of a concentration dependence of the circular dichroic spectra in this range of concentrations suggests the absence of a second-order process such as protein–protein association giving rise to the observed circular dichroic change. However, in order to assess the potential influence of protein association on the immunochemical reactivity of BGP in the presence of calcium or on the circular dichroic spectra, we have measured the molecular weight of BGP in the presence of 10 mM Ca^{2+} in the analytical ultracentrifuge. With these sets of conditions, a molecular weight of 5680 was obtained, and this value is in good agreement with the molecular weight of bovine BGP obtained from the sequence data, 5845.

The dicarboxylic acid side chain of γ-carboxyglutamic acid introduces three more ionic charges in the molecule when the fully carboxylated protein is compared to the uncarboxylated Glu-containing equivalent. Thus, it is possible that the antibody specificity and circular dichroic spectral alterations are reflections of the influence of the increased anion content in the 49-residue peptide. In order to examine whether ionic strength alone could influence the circular dichroic spectral alterations, spectra of BGP fully carboxylated were obtained in the presence of 0.5 M NaCl and compared to the spectra obtained for the fully carboxylated protein in the presence of 0.1 M NaCl. The results of these studies indicate that high ionic strength did not significantly influence the structural transition observed between fully carboxylated BGP in the presence and absence of calcium ion. Thus, the conformational alteration observed for the protein cannot be solely attributed to the increased anionic nature of the carboxylated protein.

Discussion

Conformation-specific antibodies have been used to obtain further information about protein structure and molecular motion in proteins (Furie et al., 1975; Hurrell et al., 1977; Furie & Furie, 1979; Tai et al., 1980; Keyt et al., 1982; Madar et al., 1982).

The antiserum obtained from rabbit 102 appears to recognize an epitope expressed in the bovine BGP molecule that is influenced by Ca^{2+} binding to the molecule. The identical epitope is also expressed in decarboxylated BGP. However, in contrast to fully carboxylated BGP, in the decarboxylated protein the epitope is independent of the presence of Ca^{2+} in the system. These observations lead to two conclusions. First, from these results it is clear that it is not the secondary antibody interaction, i.e., goat anti-rabbit precipitation, that is influenced by Ca^{2+} binding but rather a Ca^{2+}-influenced epitope in the BGP itself. Second, the identity of binding of uncarboxylated BGP ($\pm Ca^{2+}$) and fully carboxylated BGP ($+Ca^{2+}$) indicates that the carboxylation of the BGP molecule alters the protein such that the epitope is only expressed in the presence of divalent cation. In the present study, we have used Ca^{2+} and Mg^{2+} to effect the conformational transition required for antibody binding to the fully carboxylated bovine BGP; however, it is likely that the other cations, which have been shown to interact with vitamin K dependent blood-clotting proteins, will likely also effect this transition.

The results obtained with the Ca^{2+}-related epitope indicate identity between decarboxylated BGP ($\pm Ca^{2+}$) and carboxylated BGP ($+Ca^{2+}$). However, studies of circular dichroism of the bovine BGP in the carboxylated and decarboxylated state in the presence and absence of calcium ion indicate that the similarities between the decarboxylated protein and the carboxylated protein in the presence of divalent cation are more extensive than those simply related to the epitope associated with Ca^{2+} binding. Qualitative and quantitative interpretation of the circular dichroic spectra would suggest that the introduction of the three carboxyl groups, as a consequence of vitamin K action during synthesis of bovine BGP under normal conditions, introduces a significant conformational alteration in the molecule when compared to the uncarboxylated species. The conformational alteration that occurs upon carboxylation, while perhaps influenced by the increased anion content of the carboxylated protein, cannot be dominated by this feature since the circular dichroic spectra are not appreciably influenced by significant increases in ionic strength of the medium.

The circular dichroic spectral alterations that occur upon decarboxylation of bovine BGP can be contrasted with the results obtained for the vitamin K dependent prothrombin fragment 1, which contains 10 γ-carboxyglutamic acid residues. Upon decarboxylation of that protein (Poser & Price, 1979; Tuhy et al., 1979), virtually no change occurs in the circular dichroic spectra relative to the fully carboxylated prothrombin fragment 1 in the absence of Ca^{2+}. When Ca^{2+} is added to fully decarboxylated prothrombin fragment 1, no spectral alteration is obtained, while for carboxylated bovine prothrombin fragment 1, a significant spectral shift is observed. Thus, for the Gla-containing prothrombin fragment 1 studies, the decarboxylated species is the most similar to the carboxylated species in the absence of calcium ion; virtually the opposite result is seen in the present study. This comparison, however, must also include the fact that the Gla residues in prothrombin fragment 1 are only found in the NH_2-terminal 32 residues or in about the first fifth of the molecule. In bovine BGP the three Gla residues are located near the middle of the sequence.

Hauschka and Carr have studied circular dichroism, fluorescence, and UV spectral alterations for chicken BGP in the presence of Ca^{2+} and after decarboxylation of the protein. Chicken BGP differs from bovine BGP at approximately 16 of the 49 amino acid residues (of the bovine protein). For the decarboxylated species, these authors obtained an *apparent* value of 18% α-helix in the absence of calcium ion and 26% α-helix in the presence of calcium ion, while for the carboxylated species, Ca^{2+} changes the relative fraction of α-helix from 8% to 38%. Thus, one might conclude from their results, as well as ours, that the decarboxylated species lay somewhere between the structures of the native protein in the absence of calcium ion and the native protein in the presence of calcium ion.

Bovine BGP appears to contain three Ca^{2+}-binding sites with an average K_d of approximately 3 mM (Price et al., 1977). In the present study, the Ca^{2+} dependence of antibody binding, which we infer to be a reflection of the expression of the Ca^{2+}-dependent epitope, occurs with a transition midpoint of approximately 1.75 mM. The antibody studies were conducted using nanogram amounts of protein; hence, it is unlikely that the concentration of protein added significantly influenced the nominal Ca^{2+} concentration. Thus, it is likely that the Ca^{2+} dependence of the antibody-selected transition occurs with a K_d of approximately 1.75 mM. Considering the overall precision of Ca^{2+}-binding data in the millimolar range using equilibrium dialysis techniques, it is likely that the transition we observed represents the event studied by Price et al., which involves filling all three sites.

The data presented in this paper may be summarized by a model in which the epitope toward which the antibodies are directed either is not presented or is inaccessible in the fully carboxylated protein in the absence of Ca^{2+}. Decarboxylation of the protein to give rise to glutamate residues provides for, or permits presentation of, the epitope. For the carboxylated species, filling of the Ca^{2+}-binding site accomplishes the same phenomenon. The overall structural alteration for the required conformational change is probably in the range of a 5% increase in the *apparent* α-helical content resulting from decarboxylation or addition of Ca^{2+} to the fully carboxylated protein. Although the circular dichroic spectra of decarboxylated BGP ($\pm Ca^{2+}$) are similar to the circular dichroic spectra of fully carboxylated BGP in the presence of calcium ion, the two structures are not identical. Thus, although the epitope presentation is identical for decarboxylated BGP ($\pm Ca^{2+}$) and carboxylated BGP ($+Ca^{2+}$), other subtle alterations also occur in structure.

The overall *apparent* α-helical content observed for the fully carboxylated protein in the presence of calcium ion for the bovine protein is on the order of 14% and is therefore consistent with the α-helical derived model of BGP binding to hydroxyapatite suggested by Hauschka and Carr. In this model the Ca^{2+}-binding sites of chicken BGP are presumed to be oriented physically by the large content of α-helix in chicken BGP (38%) so as to orient the face of the molecule in appropriate juxtaposition to the surface of a hydroxyapatite crystal. Even though the apparent level of α-helix present in bovine BGP is smaller than that observed for chicken BGP, it may be sufficient to provide for the predicted structural hypothesis for the chicken protein by Hauschka and Carr. It is also likely that the same γ-carboxyglutamic acid residues are involved in the metal ion binding by BGP and in the binding of BGP to hydroxyapatite.

The interpretations for potential structures using the Chou & Fasman (1978) parameters, made by Hauschka and Carr for BGP and by Nelsestuen and co-workers for prothrombin fragment 1 and the other vitamin K dependent proteins involved in blood clotting, are based on the assumption that the γ-carboxyglutamate side chains are strongly helix forming and equivalent to glutamate side chains. While this assumption might appear reasonable in view of the lack of explicit protein models involving Gla, it is curious to note that, for both BGP and prothrombin fragment 1, binding of calcium ions presumably by the γ-carboxyglutamate residues in each case involves a conformational transition in which the *apparent* content of α-helix increases in these proteins. Indeed, decarboxylation of BGP also results in an increase in an *apparent* α-helix content of the protein. These observations permit the suggestion that the Gla side chain may, in fact, interfere with the formation of regular-ordered α-helical structures and that the binding of Ca^{2+} to a γ-carboxyglutamate residue transforms it into a more glutamate-like side chain that can participate in helices.

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Registry No. Calcium, 7440-70-2; magnesium, 7439-95-4.

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