ON THE ROLE OF γ-CARBOXYGLUTAMIC ACID IN CALCIUM AND PHOSPHOLIPID BINDING.

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Summary

The vitamin K-dependent amino acid, γ-carboxyglutamic acid, is essential for calcium binding by prothrombin and blood clotting factor X. The studies reported here demonstrate that while γ-carboxyglutamic acid-containing peptides will bind calcium, a secondary/tertiary protein structure is also necessary to form the tight calcium binding sites which are required for binding these proteins to phospholipid surfaces.

Introduction

It has recently been demonstrated that the modified amino acid, γ-carboxyglutamic acid, is found in the vitamin K-dependent blood-clotting proteins (1-3) and is necessary for calcium and phospholipid binding by these proteins (4-7). Studies have demonstrated that there are 10 γ-carboxyglutamic acid residues in the amino terminal region of prothrombin and a similar number in the amino terminal region of factor X (3,8,9). Sequence analyses have indicated that these residues are found primarily in pairs (3,7,10,11) and it has been proposed that the cluster of 4 carboxyl groups provided by this structure is responsible for calcium binding by these proteins. Other proposals based on model building involve calcium-binding through a complex arrangement of the carboxyl groups (3). The present study supplements the previous reports of calcium binding by these proteins (12-16) by providing some limitations for the role of γ-carboxyglutamic acid residues in calcium binding and in the subsequent binding of these proteins to phospholipid.

Materials and Methods

The preparation of bovine prothrombin (4), fragment 1 (17), bovine factor X (3), and the vitamin K-dependent peptides of prothrombin[1] (4) and factor X (3), as well as the method of disulfide bond reduction and ^{14}C-carboxamidomethylation (4), are described previously. [^{3}H]Sialyl-fragment 1 was produced by the method of Van Lenten and Ashwell (18) and tritium-labelled vitamin K-dependent peptide was generated by the Wilzboch procedure performed by the Amersham/Searle Corp. on 30 mg of peptide. The repurified peptide contained 0.1 mCi/mg and was added in tracer amounts to untreated peptide in order to quantitate the vitamin K-dependent peptide in the calcium binding studies.

Calcium binding studies were performed by gel-filtration using a Sephadex G-25 column (0.6 x 20 cm) equilibrated with the appropriate $^{45}CaCl_2$ (25,000 cpm/μmole) concentration in 0.05 M Tris buffer (pH 7.5)-0.1 M NaCl at 25 ± 2°. Protein concentration was determined using an $\varepsilon^{1\%}_{280} = 10.1$ for fragment 1 (molecular weight 25,000) (17) and an $\varepsilon^{1\%}_{280} = 16.5$ for prothrombin (molecular weight = 72,000) (19). Calcium was quantitated by radioactive scintillation counting. A small amount of [^{3}H]peptide (above) was added to the vitamin K-dependent peptides to obtain a known specific radioactivity allowing $^{45}Ca^{2+}$ and peptide concentrations to be determined by dual-label counting. The quantities of material applied to the column for individual determinations were: prothrombin, 7 mg, fragment 1, 2-4 mg, prothrombin vitamin K-dependent peptide, 0.2-1.0 μmoles or factor X vitamin K-dependent peptide, 0.1 μmoles.

Adsorption of proteins and peptides to phospholipid vessicles was performed according to Esmon et al. (5) using cephalin obtained from Sigma Chemical Co. as the phospholipid. The 1.2 x 50 cm Sephadex G-100 column was equilibrated with 0.05 M Tris buffer (pH 7.5)-0.1 M NaCl containing either 2 mM $CaCl_2$ or 2 mM EDTA. Other conditions for binding are as described by Esmon et al. (5).

Results and Discussion

The vitamin K-dependent structures of prothrombin, γ-carboxyglutamic acid residues, are essential for the calcium binding and barium citrate adsorbing properties of that protein. The barium citrate adsorption property apparently requires a specific arrangement of some of the γ-carboxyglutamic acid residues as evidenced by the fact that only one tryptic peptide from prothrombin, which contains eight γ-carboxyglutamic acid residues, will adsorb to barium citrate (4) whereas a second peptide with two γ-carboxyglutamic acid residues (2) is not isolated by barium citrate adsorption. Furthermore, the barium citrate-adsorbing property is asso-

[1]The tryptic peptides from prothrombin and factor X which adsorb quantitatively onto barium citrate and contain most of the γ-carboxyglutamic acid residues are referred to as "vitamin K-dependent peptides". The products of thrombin action on prothrombin are referred to as fragment 1 and intermediate 1 (5).

ciated with a nonapeptide (8) which contains only four of the γ-carboxyglutamic acid residues.

Determination of the structure necessary for calcium binding was examined by evaluation of calcium binding by the vitamin K-dependent peptide. The methods previously used for studying calcium binding by this peptide (12) subsequently have been reported to give erroneous results (21). The results obtained by the gel filtration procedure used here are shown in Figure 1a. The important aspects of calcium binding by the peptide are: 1) the affinity of the peptide is lower than that observed for prothrombin (Figure 1b), 2) although the peptide contains eight of the 10 γ-carboxyglutamic acid residues found in prothrombin, the maximum calcium bound by the peptide is about three moles/mole whereas prothrombin will bind about 10 moles of calcium per mole (13-16), 3) there is no apparent cooperativity of calcium binding to the peptide as there is for prothrombin (Figure 1b)

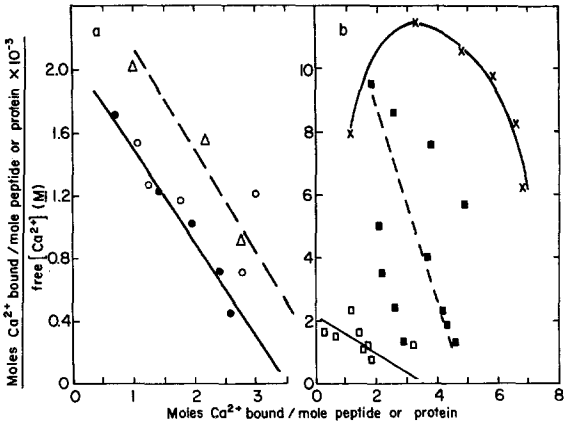

Figure 1. Scatchard plots of calcium bound by various proteins and peptides which contain γ-carboxyglutamic acid. Figure 1a gives the calcium binding observed for the vitamin K-dependent peptide with disulfide bonds intact (-●-) and with carboxamidomethylcysteine (-o-). The calcium-binding by the vitamin K-dependent peptide of factor X with disulfide bonds intact is also given (-Δ-). Figure 1b illustrates the calcium binding observed for prothrombin (-x-), for native fragment 1 (-■-) and for fragment 1 which contains carboxamidomethylcysteine (-□-).

and 4) the calcium binding by the peptide is not affected by disulfide bond reduction and derivatization which is identical to the observations with respect to barium citrate adsorption (4). Calcium-binding by the vitamin K-dependent peptide from factor X was also examined and is qualitatively the same as for the prothrombin peptide (Figure 1a, Δ). This comparison establishes that the low calcium binding observed for the prothrombin peptide is not due to the fact that the prothrombin peptide contains only eight of 10 γ-carboxyglutamic acid residues since the factor X peptide contains all of the γ-carboxyglutamic acid residues found in factor X (3).

The results of calcium-binding by prothrombin and fragment 1 using several techniques have been reported (12-16). The calcium-binding characteristics of prothrombin and fragment 1 observed in the present study are given in Figure 1b. The upward slope of the Scatchard plot observed at low calcium did not appear to be dependent on protein concentration as determined by other studies using one mg of prothrombin per determination instead of the seven mg used in Figure 1b. This apparent cooperativity of calcium binding has been observed by others (13,15) with some exception (14). Fragment 1 appears to bind less calcium than prothrombin (Figure 1b) which agrees with the results of Bajaj et al. (15) but not with those of Benson et al. (14). The observation important to the present study is that reduction and carboxamidomethylation of fragment 1 causes a substantial decrease in calcium binding affinity (Figure 1b, compare -■-, and -□-) such that the reduced carboxamidomethylated protein binds calcium substantially the same as the vitamin K-dependent peptide. For comparison, the line drawn for reduced carboxamidomethylated fragment 1 (Figure 1b, -□-) is the same as that drawn for the prothrombin vitamin K-dependent peptide (Figure 1a, -●-). Air oxidation of reduced fragment 1 did not regenerate the tight calcium-binding sites.

Previous reports indicated that disulfide bonding had only a small effect on the calcium binding by prothrombin (12) but those studies involved

a calcium binding method (20) which has subsequently been reported to give erroneous binding data (21). The failure to observe an effect of disulfide bond reduction on calcium binding in the previous studies (12) could be explained if one source of difficulty with that method were a denaturation of the protein during the procedure. In fact, the binding affinity observed in the earlier studies approximates that observed here for reduced, carboxamidomethylated fragment 1 (Figure 1b, -□-).

A second important consideration concerning the role of γ-carboxyglutamic acid is associated with its lipid-binding function. Calcium binding by the vitamin K-dependent proteins is important in blood coagulation from the standpoint of binding the proteins to a phospholipid surface where zymogen activation occurs (3,5,22,23) and it is not clear whether the calcium binding properties of the protein in solution will correlate with the role of calcium binding in the attachment of the proteins to a phospholipid surface. Therefore, the binding of fragment 1 and vitamin K-dependent peptide to phospholipid was studied to determine if the factors which are important to tight calcium binding by the protein in solution are also important for binding the protein to phospholipid. Intact fragment 1 binds to phospholipid vessicles in the presence of calcium but not in the presence of EDTA (Figure 2a and b respectively) which agrees with other studies on fragment 1 and prothrombin (5). In contrast, the vitamin K-dependent peptide (Figure 2c) does not bind to phospholipid in the presence of calcium ions. Studies using the prothrombin or factor X vitamin K-dependent peptides with intact disulfide bonds gave identical results, i.e., no binding to phospholipid. In addition, fragment 1 with reduced and derivatized disulfide bonds did not bind to phospholipid using the conditions given in Figure 2a. This establishes that secondary/tertiary protein structure is required for binding these proteins to phospholipid. The present studies demonstrate that, while γ-carboxyglutamic acid residues are involved in calcium binding, they are insufficient simply by virtue of their location in

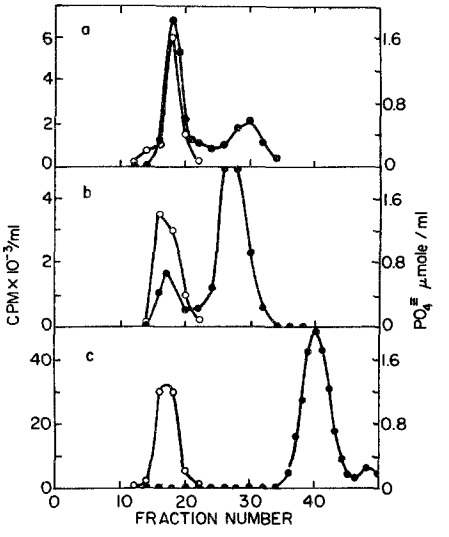

Figure 2. Binding of fragment 1 and vitamin K-dependent peptide to phospholipid. A 1.2 x 55 cm column of Sephadex G-100 was equilibrated with 0.05 M Tris buffer (pH 7.5)-0.1 M NaCl containing either 2 mM Ca^{2+} (figures 2a and 2c) or 2 mM EDTA (figure 2b). Phospholipid (0.5 ml of 6 mg/ml) in the appropriate column buffer was mixed with either fragment 1 or vitamin K-dependent peptide and incubated for 30 min. at room temperature before loading onto the column. Radioactivity (-•-), phospholipid measured as phosphate (-o-), and A_{280} (not shown) were monitored. Figure 2a shows the elution profile of [^{3}H]sialyl-fragment 1 (0.4 mg) in 2 mM Ca^{2+}; figure 2b is the same except with 2 mM EDTA substituted for Ca^{2+}; and figure 2c gives the profile of the vitamin K-dependent peptide from prothrombin labelled with ^{14}C-carboxamidomethylcysteine (0.5 mCi/mmole) from a column eluted with buffer containing 2 mM Ca^{2+}.

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a peptide sequence to provide the tight calcium-binding sites which are important in blood coagulation. These results do not eliminate the possibility that groups in fragment 1 other than the carboxyl groups of γ-carboxyglutamic acid are involved in tight calcium binding and the determination of the detailed calcium-binding structure will require x-ray crystallographic studies on fragment 1 or prothrombin.

Present evidence on the characteristics of proteins containing γ-carboxyglutamic acid and their functions indicate that this structure is not widely distributed in nature. First of all, the presence of these residues does not provide particularly tight calcium binding sites. The sites re-

quire several γ-carboxyglutamic acid residues and a specific secondary/tertiary protein structure for arrangement of these residues. The observation that most of these residues occur in pairs in the peptide sequence may have a further significance which is not yet known. Secondly, binding of protein to phospholipid through calcium ions, a function ascribed to γ-carboxyglutamic acid, can be fulfilled by other structures as evidenced by the S-100 protein from brain which binds to phospholipid through calcium ions (24) but contains no γ-carboxyglutamic acid (T. H. Zytkovicz and G. L. Nelsestuen, unpublished data). The protein-phospholipid interaction which occurs in the blood-clotting reactions and requires γ-carboxyglutamic acid even may be found to be characteristic of those reactions alone.

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References

1. Stenflo, J., Fernland, P., Egan, W. and Roepstorff, P. (1974) Proc. Nat. Acad. Sci. USA 71, 2730-2733.
2. Nelsestuen, G. L., Zytkovicz, T. H. and Howard, J. B. (1974) J. Biol. Chem. 249, 6347-6350.
3. Howard, J. B. and Nelsestuen, G. L. (1975) Proc. Nat. Acad. Sci. USA 72, 1281-1285.
4. Nelsestuen, G. L. and Suttie, J. W. (1973) Proc. Nat. Acad. Sci. USA 70, 3366-3370.
5. Gitel, S. N., Owen, W. G., Esmond, C. T. and Jackson, C. M. (1973) Proc. Nat. Acad. Sci. USA 70, 1344-1348.
6. Stenflo, J. and Ganrot, P. O. (1972) J. Biol. Chem. 247, 8160-8166.
7. Stenflo, J. (1974) J. Biol. Chem. 249, 5527-5535.
8. Howard, J. B. and Nelsestuen, G. L. (1974) Biochem. Biophys. Res. Comm. 59, 757-763.
9. Zytkovicz, T. H. and Nelsestuen, G. L. (1975) J. Biol. Chem. 250, 2968-2972.
10. Magnusson, S., Sottrup-Jensen, L., Petersen, T. E., Morris, H. R. and Dell, A. (1974) FEBS Lett. 44, 189-193.
11. Enfield, D. L., Ericsson, L. H., Walsh, K. A., Neurath, H. and Titani, K. (1975) Proc. Nat. Acad. Sci. USA 72, 16-19.
12. Nelsestuen, G. L. and Suttie, J. W. (1972) Biochemistry 11, 4961-4964.
13. Stenflo, J. and Ganrot, P. O. (1973) Biochem. Biophys. Res. Commun. 50, 98-104.
14. Benson, B. J., Kisiel, W. and Hanahan, D. J. (1973) Biochim. Biophys. Acta 329, 81-87.
15. Bajaj, S. P., Butkowski, R. J. and Mann, K. G. (1975) J. Biol. Chem. 250, 2150-2156.
16. Dombrose, F. A., Gitel, S. N. and Jackson, C. M. (1975) Fed. Proc. 34, p. 258(abs).
17. Heldebrant, C. M. and Mann, K. G. (1973) J. Biol. Chem. 248, 3642-3652.
18. Van Lenten, L. and Ashwell, G. (1971) J. Biol. Chem. 246, 1889-1894.

19. Ingwall, J. S. and Scheraga, H. A. (1969) Biochemistry 8, 1860-1869.
20. Paulus, H. (1969) Anal. Biochem. 32, 91-100.
21. Heyde, E. (1973) Anal. Biochem. 51, 61-66.
22. Barton, P. G. and Hanahan, D. J. (1969) Biochim. Biophys. Acta 187, 319-327.
23. Esmon, C. T., Owen, W. G., and Jackson, C. M. (1974) J. Biol. Chem. 249, 7798-7807.
24. Calissano, P., Alema, S. and Fasella, P. (1974) Biochemistry 13, 4553-4560.