Chemical modification of bovine prothrombin fragment 1 according to the procedure of D. J. Welsch and G. L. Nelsestuen (1988) [Biochemistry 27, 4946-4952 and earlier papers] provided a series of fragment 1 derivatives in which various nitrogen-containing side chains were N-acetylated and/or N-2,4,6-trinitrophenylated. In addition the des-[Ala-1,Asn-2]- and des-[Ala-1,Asn-2,Lys-3]-fragment 1 derivatives were prepared by limited enzymatic hydrolysis of fragment 1 using cathepsin C and plasmin, respectively. Quantitative studies on the Ca(II) binding of these proteins have been accomplished using 45Ca(II) equilibrium dialysis. Binding of these fragment 1 derivatives to phosphatidylserine/phosphatidylcholine (PS/PC) vesicles (25:75) in the presence of Ca(II) ions has been studied using the light-scattering technique.

Acylation of the 5 lysine residues of fragment 1 by the action of acetic anhydride (500-fold molar excess) in the presence of 75 mM Ca(II), pH 8.0, results in loss of positive cooperativity in Ca(II) binding (Scatchard plot) and an increase in the number of Ca(II) ions bound. The Ca(II)-dependent PS/PC binding of the acylated protein is reduced. Removal of 2 and 3 residues from the amino terminus likewise leads to loss of positive cooperativity in Ca(II) binding and reduced binding affinity to PS/PC vesicles. The important role of the amino-terminal 1-10 sequence is discussed. We conclude that positive cooperativity in Ca(II) binding is not a prerequisite for the Ca(II)-dependent binding of bovine prothrombin fragment 1 to PS/PC vesicles.

The vitamin K-dependent carboxylation of specific glutamic acid residues in the amino-terminal region of several coagulation and anticoagulation proteins forms a negatively charged domain that contains the essential γ-carboxyglutamic acid (Gla) residues (1-6). These proteins require Ca(II) for the amino-terminal 1-10 sequence is discussed. We conclude that positive cooperativity in Ca(II) binding is not a prerequisite for the Ca(II)-dependent binding of bovine prothrombin fragment 1 to PS/PC vesicles.

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1 The abbreviations used are: Gla, γ-carboxyglutamic acid; γ-MGlu, γ-methyleneglutamic acid; fragment 1, residues 1-156 of bovine prothrombin; des-[Ala-1,Asn-2]-F-1, residues 3-156 of bovine prothrombin; N-Ac-F-1, N-acetyl-Ala-1-N'-acyetyl-Lys-3,11,44,57,97-N(β-amido)-Asn-101-fragment 1; H2N-Ac-Ala-1-(N'-Ac-Lys)7-F-1, N-2,4-dinitrobenzoyl-NH2-Ala-1'-N'-acyetyl-Lys-3,11,44,57,97-fragment 1; PTH, phenylthiohydantoin; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PS/PC; phosphatidylserine/phosphatidylcholine; HPLC, high-performance liquid chromatography; PL, phospholipid.

2 Portions of this paper (including "Experimental Procedures, part of "Results," Fig 1, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
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The calculated binding constants (Table II) were obtained from reciprocal plots of 1/ν vs. 1/[protein]max (Appendix I). (ν = moles of protein bound/moles of phospholipid). The phospholipid binding isotherms are shown in Figs. 4 and 6. Values for N (moles of protein bound/mole of phospholipid) are also listed for each modified protein (Table II). Error bars indicate the 95% confidence level as obtained by SAS NLIN option analysis of the reciprocal plots from separate determinations.

Removal of 2 (entry 6, Table II, and Fig. 5) or 3 (entry 7, Table II) residues from the amino terminus likewise results in a decrease in Ca(II) affinity as evidenced by the reduced kobs values and loss of positive cooperativity in Ca(II) binding. However the number of Ca(II) sites is unchanged in the des-[Ala-1,Asn-2,Lys-3] analog (entry 7, Table II) and Ca(II)-dependent PS/PC binding is still observed. The Kd of des-[Ala-1,Asn-2]-F-1 (entry 6) is lower than that of the des-[Ala-1,Asn-2,Lys-3]-F-1 derivative. Thus loss of 2 or 3 residues from the amino terminus decreases Ca(II) affinity but does not affect the PS/PC binding.

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**Table II**

| Entry | Protein | ν* | Ca(II), kobs | PS/PC, Kd | n |
|-------|---------|---|-------------|-----------|---|
| 1     | b-F-1  | 7 | Cooperative | 0.4 ± 0.1 | 0.014 |
| 2     | RCM-F-1 | 9 | (7463), (240) | ND* | |
| 3     | H2N-Ala-1-(N'-Ac-Lys)6-F-1 (b-F-1 + AcO2+Ca) | 9 | 1270 | 1.1 ± 0.02 | 0.009 |
| 4     | N'-Ac-Ala-1-(N'-Ac-Lys)6-F-1 (b-F-1 + AcO2+Mg) | 9 | 360 | ND | |
| 5     | N'-Ac-F-1 (b-F-1 + AcO2) | 8 | 450 | ND | |
| 6     | des-[Ala-1,Asn-2]-F-1 | 7 | 330 | 1.1 ± 0.5 | 0.011 |
| 7     | des-[Ala-1,Asn-2,Lys-3]-F-1 | 9 | 430 | 2.2 ± 0.04 | 0.016 |
| 8     | TNP-F-1 (b-F-1 + TNBS') | 10 | 980 | 0.9 ± 0.06 | 0.017 |
| 9     | TNP-des-[Ala-1,Asn-2]-F-1 (Entry 8 + TNBS) | 9 | 750 | 0.5 ± 0.02 | 0.016 |
| 10    | TNP-des-[Ala-1,Asn-2,Lys-3]-F-1 (Entry 7 + TNBS) | 10 | 610 | 0.7 ± 0.01 | 0.010 |
| 11    | TNP-Ala-1-(N'-Ac-Lys)6-F-1 (Entry 3 + TNBS) | 8 | 400 | 1.1 ± 0.06 | 0.011 |
| 12    | N'-DNP-Ala-1-(N'-Ac-Lys)6-F-1 (Entry 3 + DNBHSS') | 7 | 670 | 2.0 ± 0.04 | 0.010 |
| 13    | TNP-F-1 + AcO2 + Ca | 8 | 740 | 2.1 ± 0.03 | 0.010 |
| 14    | TNP-F-1 + AcO2 | 7 | 720 | ND | |

* Errors are ±1.0 site.
* Errors are ±20%.
* Data reported in Ref. 11.
* AcO2, acetic anhydride.
* ND, none detected (detection limits for this assay are ±25 μM under the conditions used).
* DNBHSS, 2,4-dinitrobenzoic acid N-hydroxysulfosuccinimide ester.
not abolish Ca(II)-dependent PS/PC binding. Acylation of
des-[Ala-1,Asn-2]-F-1 with acetic anhydride in the presence of
Ca(II) yields a modified protein which in contrast to
fragment 1, cannot be sequenced indicating that Lys-3 is fully
acylated.

Treatment of fragment 1 with excess TNBS (pH = 9.9)
yields TNP-F-1 (entry 8, Table II, and Fig. 3). The protein
exhibits decreased Ca(II) affinity relative to H2N-Ala-1-(N"Ac-Lys),-F-1 (entry 3) and contains 10 Ca(II) sites which are
equivalent and noninteracting. Despite the loss of the electron
donor properties of the NHg, the TNP-F-1 binds to PS/PC
vesicles with approximately the same affinity as entry 3.

FIG. 4. Phospholipid binding isotherms for native b-F-1 (□),
TNP-F-1 (■), H2N-Ala-1-(N"Ac-Lys),-F-1 (△), N"Ac-F-1 (●).

FIG. 5. Ca(II) ion binding Scatchard plots of des-[Ala-
1,Asn-2]-b-F-1 and (inset) TNBS-treated des-[Ala-1,Asn-2]-
F-1.

FIG. 6. Phospholipid binding isotherms for native b-F-1 (□),
des-[Ala-1,Asn-2]-F-1 (△), and TNBS-treated des-[Ala-1,
Asn-2]-F-1 (●).

try 10) were examined. Both TNP-proteins displayed improved PS/PC binding over the corresponding NHg-free an-
logs. Treatment of entry 3 with TNBS yielded TNP-Ala-1-(N"Ac-
Lys),-F-1 with 2,4-dinitrobenzoic acid N-hydroxysulfos-
uccinimide ester to obtain the N-2,4-dinitrobenzoylester derivative
(entry 12, Table II). In contrast to H2N-Ala-1-(N"Ac-Lys),-
F-1 (entry 4), this modified protein, N"DNB-Ala-1-(N"Ac-
Lys),-F-1, bound to PS/PC vesicles with a low affinity and
exhibited a Ca(II) binding stoichiometry and affinity that
were not unlike TNP-Ala-1-(N"Ac-Lys),-F-1 (entry 11).

Acylation of TNP-F-1 in the presence of Ca(II) ions was
expected to lead to little change in the properties of the product. However, the resulting product (entry 13, Table II)
exhibited an apparently reduced Ca(II) stoichiometry and a
reduced PS/PC affinity relative to entry 9 or 8. As expected
the reaction of TNP-F-1 with acetic anhydride in the absence of
Ca(II) (entry 14, Table II) abolished PS/PC binding. The
Ca(II) stoichiometry and k₄₅ values of entry 5 and 14 were
similar. Thus acylation of fragment 1 (entry 5) or the TNP-
F-1 derivative (entry 14, Table II) in the absence of Ca(II)
ions yields proteins that are quite similar in Ca(II) binding
properties to reduced and carboxymethylated fragment (com-
pare entries 5 and 14 to entry 2, Table II).

**DISCUSSION**

The principal focus of this study was to quantitatively
evaluate the effects of chemical modification at the amino
groups of bovine fragment 1 on Ca(II) and PS/PC binding.
Several conclusions regarding the "Ca conformation" required
for Ca(II)-dependent binding of fragment 1 to PS/PC vesicles
emerge from this study.

We have previously suggested that the positive cooperativity
observed in Ca(II) binding to bovine fragment 1 and other
vitamin K-dependent proteins was a prerequisite for the
Ca(II)-conformation required for Ca(II)-mediated binding to
PS/PC vesicles. We based this argument on: 1) the absence
of observed cooperativity in ²⁰Mg(II) binding to fragment 1
(23) and the lack of PS/PC binding by the fragment 1-Mg(II)
complex; and 2) on the properties of T7,8,33-γ-MGlu-F-1 (14).

The latter studies indicated that the ²⁰Mg(II) binding to the
7,8,33-γ-MGlu-F-1 was identical to ²³Mg(II) binding to frag-
ment 1. The metal ion-promoted quenching of the intrinsic
fluorescence was retained by the 7,8,33-Gla-modified protein;
however this protein had lost positive cooperativity in Ca(II)
binding, exhibited reduced Ca(II) affinity and, importantly,
did not bind to PS/PC vesicles. The acylation studies of
Welsch and Nelsestuen (15-19) that lead to H2N-Ala-1-(N"Ac-
Lys),-F-1 upon acylation in the presence of Ca(II) ions and
N"Ac-Ala-1-(N"Ac-Lys),-F-1 in the presence of Mg(II)
ions also support the idea of different conformational popu-
lations induced by the interaction of different ions (calcium
and magnesium with different charge densities, coordination
states, and ligand preferences) with fragment 1. Nevertheless,
it is clear from the data reported in Table II that positive
cooporativity in Ca(II) binding to the various fragment 1
derivatives is not a precondition reflective of, or required for,
PS/PC binding. Furthermore, the Ca(II) affinity of the par-
ticular protein does not appear to be a good predictor of PS/
PC binding. For example, des-[Ala-1,Asn-2]-F-1 (entry 6,
Table II) exhibits a k₄₅ value of 330 M⁻¹ which is quite similar
to that of $N^\alpha$-Ac-Ala-1-(N$^\alpha$-Ac-Lys)$_2$-F-1 (entry 4, Table II). However, the former protein binds to PS/PC vesicles, whereas the latter does not. Thus the ability of fragment 1 to exhibit Ca(II)-mediated phospholipid binding must depend on conformational features that are mediated by the Ca(II) binding process.

Positive cooperativity in Ca(II) binding to bovine fragment 1 involves three of the seven Ca(II) sites (23). The positive cooperativity is abolished when the amino-terminal region of the protein is modified. For example, positive cooperativity of Ca(II) binding is abolished by: removal of 2 or 3 residues from the amino terminus; modification of Gla residues 7 and 8; introduction of a N$^\alpha$-TNP or N$^\alpha$-acyl group at the amino terminus; or acetylation of the N$^\alpha$-amino groups of the 5 Lys residues. Since positive cooperativity is not observed in Mg(II) binding to the protein, the phenomena must involve some property of Ca(II) ions as well as some property of the ligand (fragment 1).

Hodgson et al. (32, 33) have suggested that the major reason for the presence of Gla rather than Glu in the family of Ca(II) binding coagulation proteins is the availability in the former of the additional carboxylate group. Hodgson et al. note that the additional carboxylate permits polymeric arrays of Ca(II) ions in the crystallographic structures of malonate complexes with Ca(II) or Ba(II). Malonate complexes with smaller metal ions such as Mg(II) or Be(II) are invariably monomeric. Polymeric arrays were also observed by Zell et al. (37) in their Ca(II) α-ethylmalonate crystal study.

One might expect to observe positive cooperativity in the formation of a polymeric system involving two or more Ca(II) ions that interact through a network of carboxylate ligands. The positive cooperativity observed with Ca(II) binding to fragment 1 might reflect the formation of such a polymeric matrix involving three Ca(II) ions. Each Ca(II) ion would be bound to two or more Gla carboxylate groups in hepta- or octacoordinate arrays as observed by Hodgson et al. (32, 33). Each Ca(II) ion would interact with the negative ligands (i.e. Gla carboxyates) positioned by the previous Ca(II) ion leading to the observed positive cooperativity associated with Ca(II) binding by the native protein.

The negatively charged Gla carboxylates in the protein can be stabilized by Ca(II) ions and/or by salt bridges with available amino groups. Chemical modification of the amino groups will affect salt bridge formation and might also affect interactions between the bound Ca(II) ions. Thus, acetylation of fragment 1 in the presence of Ca(II) ions might be expected to yield a modified protein (entry 3, Table II) which retained the essential Ca(II)-promoted interactions between Ca(II) ions, amino groups, and the Gla carboxylate groups. Viewed in this way, the α-NH$_2$ group of Ala-1 must be an integral feature of the polymeric Ca(II)-Gla domain matrix. The involvement of the α-NH$_2$ group of Ala-1 in the positive cooperativity of the Ca(II) binding process is evident from a comparison of fragment 1 (entry 1, Table II) with either des-[Ala-1,Asn-2]-F-1 (entry 6, Table II) or des-[Ala-1,Asn-2,Lys-3]-F-1 (entry 7, Table II). Removal of Ala-1 abolishes the cooperativity of Ca(II) binding. The involvement of the ε-NH$_2$ groups of Lys residues in the Ca(II) binding process is seen by comparison of the Ca(II) binding stoichiometry of the N$^\alpha$-acytetyl- or N$^\alpha$-TNP fragment 1 derivatives. Modification of the ε-NH$_2$ groups leads to an increase in Ca(II) ions bound by Gla carboxylate groups as the Gla-ε-NH$_2$ salt bridges are abolished. Cooperativity in Ca(II) binding is lost in all cases suggesting that Gla-Lys salt bridges are involved in this event as well.

Church et al. (34, 35) have described a murine monoclonal antibody, H-11, which binds to a conserved epitope involving Phe-5 in the bovine prothrombin sequence. Binding of H-11 is inhibited by Ca(II), Mg(II), and Mn(II) ions. Church et al. (34, 35) conclude that Phe-5 is buried and thus inert to H-11 upon divalent metal ion binding to bovine fragment 1. Thus the amino-terminal 1–10 sequence must adopt an ordered conformation as a result of divalent ion binding. This proposal is supported by the present studies which indicate that subtle changes in the 1–10 region such as modification of Ala-1, acetylation of des-[Ala-1,Asn-2]-F-1 or des-[Ala-1,Asn-2,Lys-3]-F-1, and conversion of Gla-7,8 to γ-methyleneglutamyl residues can abolish or modify PS/PC binding by the protein.

We wished to demonstrate that the PS/PC binding exhibited by the TNP derivatives of the various fragment 1 derivatives was in fact a Ca(II)-dependent process. Two control experiments indicate the Ca(II) requirement. In the first experiment EDTA was added to the Ca(II)-TNP-F-1:PS/PC binding complex. The complex immediately dissociated. The second control utilized the modified protein, 10-γ-MGlu-F-1, (14) in which all Gla residues are modified. This protein does not bind to PS/PC vesicles in the presence of Ca(II) ions. Treatment of this protein with excess TNBS, isolation of the TNP-10-γ-MGlu-F-1, and incubation with Ca(II) and PS/PC vesicles gave no indication of protein-phospholipid binding. Thus the binding observed by incorporation of a TNP residue at Ala-1 or Lys-3 or Gly-4 in the appropriate fragment 1 derivative must reflect the importance of the Ca(II):Gla interactions in establishing the phospholipid binding conformation. The effect of a dinitro- or trinitrophenyl substituent at the amino terminus on PS/PC binding also suggests the presence of an ordered conformation in the 1–10 region of the protein. Recent studies by Schwabe et al. (36) indicate that Ca(II) but not Mg(II) protected the amino terminus of the amino-terminal peptides from human II (residues 1–41 and 1–44, 60:40, v/v), bovine X (residues 1–44), and bovine IX (residues 1–42). The introduction of a TNP group at the amino-terminal residue of these peptides also promoted Ca(II)-dependent binding of the peptides to PS/PC vesicles.

Acylation of fragment 1 in the presence of Mg(II) or Ca(II) protected the β-amido nitrogen atom of Asn-101 from imide formation. Acylation in the absence of metal ions leads to the production of the Asn-101 imide and perhaps modification at other sites. Acylation under these conditions results in the loss of the intrinsic fluorescence transition and PS/PC binding. Evidence suggesting a role for a non-Gla domain metal ion binding site in this region of the kringle domain is presented in the accompanying manuscript (30).

APPENDIX

Derivation of a Model of a Single Class of Noninteracting Sites for Protein Binding to Phospholipid Vesicles

1. $v' = \text{protein molecules bound}$
2. $K_a = [\text{protein}][\text{PL}].$
3. $h = \frac{[\text{protein}][\text{PL}]}{[\text{protein}][\text{PL}][\text{protein}]}.$
4. $hN = v'$, where $N$ is the moles of protein bound/mol of PL at saturation.

5. $h = \frac{v'}{N} = \frac{K_h\text{protein}}{1 + K_h\text{protein}}$

or

$N = \frac{1}{K_h\text{protein} + 1}$

Therefore,

$\frac{1}{v'} = \frac{1}{NK_h\text{protein}} + \frac{1}{N}$

6. Plot of $1/v'$ versus $1/[\text{protein}]$ gives $N$ and $K_h$. For a typical strong binder $1/N = 71.42; K_h = 2.5 \times 10^8 \text{M}^{-1}; K_p = 0.4 \mu M$.

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Ion Binding Studies on Modified Bovine Fragment 1 Proteins

Introduction

This research was motivated by the need to understand the binding properties of modified bovine fragment 1 proteins. The initial studies were performed on native fragment 1, followed by modifications to explore the effects of these changes on binding. The modified proteins were then tested for their ability to interact with target molecules, providing insights into potential applications.

Methods

Protein Purification

Native fragment 1 was purified from bovine intestine using chromatography techniques. Modified fragments were produced by chemical or enzymatic modifications. The purity of each protein was confirmed by SDS-PAGE and mass spectrometry.

Binding Assays

The binding of each modified fragment 1 was assessed using a radioligand binding assay. This involved incubating the protein with a radiolabeled ligand and measuring the amount of bound ligand using a scintillation counter.

Results

Initial experiments demonstrated a significant increase in binding affinity for the modified fragment 1 compared to the native protein. Further experiments revealed that the specific modifications enhanced the selectivity for certain target molecules.

Discussion

These findings suggest that modified fragment 1 proteins could be developed for use in therapeutic or diagnostic applications. Further work is needed to optimize the design process and to test the long-term stability and efficacy of these novel peptides.

Conclusion

In conclusion, the study of modified fragment 1 proteins provides a promising avenue for the development of new therapeutic agents. Further research is needed to fully realize the potential of these molecules.